Identification of *Giardia duodenalis* in Environmental and Animal Samples in Scotland with Development of Novel Approaches of Filtration Elution

Ben Horton

MSc by Research

Institute of Biological Chemistry, Biophysics and Bio-engineering.

Heriot-Watt University

September 2016

Abstract

Giardia duodenalis is a waterborne flagellated protozoan parasite known to cause substantial cases of disease throughout the world. The parasite is argued to be zoonotic, and as such consumption of water contaminated by animal faeces containing parasite cysts is thought to lead to human infection. Infection is skewed towards the developing world, but outbreaks do occur within the developed world. This project had two aims: to identify the prevalence of *G. duodenalis* within a range of Scottish samples, both faecal and water, and to develop a novel method for the elution of *G. duodenalis* cysts from filter matrixes by incorporating megasonic sonication into a pre-existing method – the FiltaMax system. Molecular techniques found that water samples within this study were mostly negative for parasite DNA, however faecal samples were often positive, with animal samples testing sporadically positive throughout the study. A novel methodology for filter matrix elution of *G. duodenalis* cysts was developed and proven to be comparable to current leading filtration methods. This megasonic method also boasted significant advantages over the FiltaMax system: such as reduction in labour involved, substantially reduced damage of the parasite during elution and future automation is a possibility.

Acknowledgements

I would like to thank both of my supervisors, Dr Frank Katzer and Dr Helen Bridle for all of their support, expertise and advice which was given throughout the duration of this research project. I would also like to thank Moredun Scientific who made this project a possibility via both funding and allocation of time within my working week to complete project work.

Finally, my thanks also go to staff within the protozoology division of The Moredun Research Institute for their helpful nature during this project, particularly Clare Hamilton, Jackie Thompson and Paul Bartley.

Contents

1. Chapter 1 – Introduction

1.1 *Giardia* Infection, Epidemiology and Assemblages

Giardia as a genus consists of several distinct species which are all known to have specific host ranges, with several being very narrow and others being widespread in terms of host range. Specifically, only *G. duodenalis* is of concern with regards to public health, as only this species is known to infect humans. However, it is also the species of the genus with the widest host range, thus has the potential to be zoonotic. The answer to the question, "is this parasite zoonotic", has been a cause of much debate since the discovery *of G. duodenalis* as an intestinal protozoon of man (Feng & Xiao, 2011). Infection with the parasite causes a wide range of symptoms with varying severity, something which is increasingly thought to be caused by parasite and host interaction as noted by Jerlström-Hultqvist *et al* (2010). Ten years ago the parasite was added to the Neglected Diseases Initiative, highlighting the requirements for a more comprehensive understanding of the parasite and its epidemiology.

Giardiasis patients often experience acute symptoms such as nausea, headaches, fevers, stomach cramping, flatulence and bouts of foul smelling fatty diarrhoeic stools. In a proportion of cases these symptoms disappear not long after appearing and the patient returns to seemingly normal health, although they still may be shedding the parasite in faeces for some time (termed asymptomatic infection). In other cases, symptoms may persist and become chronic, causing longer term health problems and in some cases requiring hospitalization as has been reported by Alexander *et al* (2014). These chronic symptoms have also been linked by numerous authors (Stark *et al*, 2007; D'Anchino *et al*, 2002; Halliez & Buret, 2013; Robertson *et al*, 2010) to the resemblance and possible false diagnosis of irritable bowel syndrome in individuals.

In 2013 a paper by Esch & Petersen (2013) reported *G. duodenalis* infections to be roughly $2.8x10^8$ worldwide, annually. Infection occurs when infectious cysts shed by a host contaminated the environment, leading to ingestion by a new host, via a faecal / oral cycle. Following ingestion of a cyst by a new host the parasite reproduces quickly within the gut causing exponential multiplication. New cysts of the parasite are then formed which are shed in the faeces of the host, contaminating the environment (Adam, 2001). A relatively low infective dose of between 10-100 cysts are required to cause human infection (Roxstrom-Lindquist *et al*, 2006) and this, combined with vast

numbers of cysts excreted per gram of stool, give the parasite a high potential for outbreaks if left unchecked in host populations. There is also warranted speculation, as previously mentioned, to whether the parasite could be of a zoonotic nature (Sprong *et al*, 2009; Feng & Xiao, 2011; Stuart *et al*, 2003 & Thompson, 2000 & Esch & Peterson, 2013), causing further concerns.

The protozoan parasite is among a number of other organisms referred to and recognised as 'waterborne' pathogens, such as *Cryptosporidium* species, which are also well documented to cause gastrointestinal disease in humans, through drinking or eating food which has been washed with a contaminated water source (Smith *et al*, 2007; Savioli *et al*, 2006). Due to this, the worldwide prevalence of *G. duodenalis* infection in humans is largely skewed towards developing countries with poor hygiene and inadequate water filtration or sewage controls. In these areas of the world parasite this causes a massive burden on infected individuals, often children, who will fail to thrive due to the effects of malnourishment (Clayton & Waite, 2012). Despite this, outbreaks of the disease in humans still occasionally occur in the developed world as reviewed by Barwick *et al*, (2000); Feng & Xiao (2011); Smith *et al* (2006); Lisle & Rose (1995); Daly *et al* (2010) and Cacciò & Ryan (2008). Developed world outbreaks tend to occur when water control procedures fail, leading to localized contaminated water infecting the population who consume the contaminated drinking water (Karanis *et al*, 2007).

Infection is well known to be acquired often when travelling to developing areas of the world where the parasite is common due to poor sanitation, as has been reported by many authors including, but not limited, to Ekdahl & Andersson (2005) and Alexander *et al* (2014). Numerous outbreaks have been documented throughout the world, as reviewed by Karanis *et al* (2007), which shows that where preventative measures fail the parasite can quickly thrive in the at risk population. These outbreaks highlight the need for continuous efforts to both identify and control potential sources of environmental contamination which could result in eventual human infection with the parasite. Continual work towards this goal will certainly contribute to limiting future outbreak events or, at the very least, identify where they are most likely to occur in the future and allow the implementation of appropriate preventions.

The threat of *G. duodenalis* is further amplified by a lack of understanding of its population structure; something in which progress is still hampered today. By investigating genetic distance the parasite genus duodenalis was found to consist of seven distinct genetic groups, termed 'assemblages' which range from 'assemblage A'

to ‘assemblage H’. Each of these assemblages have different host ranges which they can infect, some being broad and others narrow. Assemblages A and B have a much wider host range than the other assemblages, which has been an argument for the zoonotic status of this parasite as it also includes humans (Ryan & Cacciò, 2013). Within these two human infective assemblages there are also further defined sub-assemblages, which appear to cause disease differently and infect specific hosts more or less frequently, despite being able to infect a range of hosts. These sub-assemblages are however somewhat out with the scope of this work and so will not be discussed in depth and only referred to where necessary for better understanding. However understanding the epidemiology of these sub-assemblages is paramount in the battle against human giardiasis.

1.2 Giardiasis in the UK

In the United Kingdom, Public Health England and Wales estimated that there were 3624 reported cases of giardiasis in the population (Gov.uk, 2016) in 2013. In Scotland there were only 167 reported cases of giardiasis in the population in the same year (Hps.scot.nhs.uk, 2016). Interpretation of the data shows that there is a difference between *G. duodenalis* epidemiology within Scotland compared to that of England and Wales. Increased infection numbers within England and Wales would be expected, due to a much larger population than that of Scotland (England has almost 10 times that of the population found in Scotland alone, as of 2014 (Ons.gov.uk, 2016)), however *G. duodenalis* infection rates per head within the Scottish population is lower than that of England and the reasons for this difference are still unknown.

G. duodenalis is a notoriously difficult disease to both identify as a cause of symptomatic disease, and to accurately quantify its magnitude of infection within the populace. There are many reasons why this is the case, firstly: cases of *G. duodenalis* infection often differ in symptoms between patients, ranging from developing acute, chronic or totally non-symptomatic infection. Additionally, assemblages A and B (the main human infective assemblages and thus of main interest to public health) have been documented to cause differing severities of infection in human hosts (assemblage A causing a milder infection, B causing a more symptomatic one) (Alexander *et al*, 2014). It has also been suggested by authors that host-parasite interaction has a large impact on the symptoms that an individual will experience when infected with the organism,

which could be a contributing factor to explain the differences in clinical symptoms observed in infected patients. UK Health Protection authorities rely on the ability of their medical staff to identify, report and document disease episodes of notifiable diseases to create an idea of disease epidemiology within their respective regions. The nature of this means that there are regional biases and thus it is impossible to understand the true epidemiology of a disease; however they do give a good indication of levels of disease within a population. Unfortunately in the case of *G. duodenalis* a large number of cases may be mild, short-lived or even asymptomatic and will therefore go unreported to public health authorities in the UK, rendering them invisible to authorities. Large time delays between infection, consultation of general practitioners, provision of diagnostic samples and actual diagnosis can also lead to delayed/missed diagnoses (Cacciò & Sprong, 2011). Medical personnel may also fail to identify the parasite symptoms and miss-diagnose, whilst infected individuals may not see the need to consult a GP for short term health issues (diarrhoea etc). These patients will more than likely still be shedding the parasite in their faeces (*G. duodenalis* follows a faecal/oral lifecycle) whilst being unaware and possibly spreading disease. Further information on diagnosis, detection and treatments of the parasite will be detailed in a further section. These factors in combination undoubtedly lead to large scale under-reporting of human infective *Giardia* infections within both the UK, as well as the world in general and highlight the problematic epidemiology of the parasite (Wensaas *et al*, 2009; Breathnach *et al*, 2010; Ankarklev *et al*, 2010; Alexander *et al*, 2014).

Data suggests that the numbers of cases of giardiasis in Scotland have been slowly falling since 2008 (http://www.documents.hps.scot.nhs.uk/giz/10-year-tables/giardia.pdf); however a personal communication has suggested that in recent months this number has been increasing steadily, with unknown cause (HPS Scotland, Oct 2015).

Residing in the developed world, Scotland and the overall UK population enjoy very high quality drinking water that is expected with its high standard of living. Public health figures however do show that although the UK has effective water treatment systems and controls, which are vigorously regulated by officials, there is still a threat from the parasite which occasionally manifests in localized outbreaks in the population. In order to identify and understand potential sources of disease outbreaks within the UK, understanding of local catchments and potential for contamination is paramount. Transmission of the parasite within recreational waters such as swimming baths has also

been seen worldwide (Porter *et al*, 1988; Katz *et al*, 2006); however this will not be focused on in this project. It has been previously highlighted that livestock pasture are significant sources of contamination of water sources worldwide (as reviewed by Plutzer *et al*, 2010) something which will be true for the UK or indeed Scotland. *Giardia* cysts in contaminated water sources as well as companion animals are thought to be an important reservoir for human infection (Slifko *et al*, 2000). Scotland, and indeed the UK in general, has always had a rich history of farming practice and large areas of uninhabited countryside, notably in the Scottish highland areas, used for grazing livestock. As such one would expect that an increased level of *G. duodenalis* would be found within these countryside areas compared to other, more urbanised parts of the UK. Furthermore the contamination of these water sources by grazing animals with *Giardia* cysts is a threat which is known to occur as water has been highlighted countless times as a main route of human infection with the parasite (Baldursson & Karanis, 2011; Slifko *et al*, 2000). Due to investigations into both the presence and specific assemblages of *Giardia* cysts in livestock reservoirs, especially which border catchments for eventual human consumption, are critical in understanding the dynamics of infection cycles and outbreak potentials within the local areas of Scotland and the UK.

In 2001, in Denmark, the findings to a study (Homan & Mank, 2001) were also similar to previous mentioned work by Alexander *et al* (2014), with assemblage B causing more symptomatic and severe infections in comparison to assemblage A within patients. Interestingly, other work has previously shown that levels of human infection with the less severe *G. duodenalis* assemblage A are much less common worldwide when compared to assemblage B, which has been identified in the majority of reported cases of giardiasis in humans (Cacciò & Ryan, 2008). Studies carried out specifically in the UK however highlight an interesting epidemiological pattern within the countries. A study in London by Breathnach *et al* (2010) agreed with the general world-wide pattern of assemblage B prevalence in human infection, showing that infection patterns followed this rule within the London area. In Scotland however the situation could be somewhat different as Alexander *et al* (2014) reported that the occurrence of assemblage A + B in infections in Scotland was actually the reverse of the situation as reported in both London, and indeed worldwide, with assemblage A being more commonly diagnosed in infections.

A potential reason for a lower number of infections in Scotland compared to the UK in general, previously raised by Pollock *et al* (2005), was that infection with the less symptomatic assemblage A may be more common in the population. The results of this study suggest that this may indeed be the case and thus fewer reports of infection occur as the dominant assemblage (A) causes less symptomatic infections. The study by Alexander *et al* (2014) was carried out on 30 reported cases of *G. duodenalis* infection, with patient samples received over 2011-2012 and over 70% of the cases were found to be infected with assemblage A. Twenty-two of the individuals ,who were infected, had however reported travel (to high risk regions) abroad shortly before falling ill. This factor suggests that infection could have originated from abroad, however cannot be conclusively identified. The factor of travel abroad can be somewhat overlooked, as health surveillance reports within England and Wales will also include patients diagnosed which have been abroad recently, which would cancel this out as an uncontrolled variable between the studies. As the majority of the infections are found to be caused by assemblage A, this does mean that the potential for zoonotic infection could be at an increased risk in Scotland as it is known that assemblage A tends to infect a wide range of animals such as livestock, wildlife and companion animals along with humans. Assemblage B, although it has been known to infect other animals, tends to generally infect humans most commonly (Feng & Xiao, 2011). If this is the case and assemblage A dominates human infections within Scotland, further work will need to be carried out to assess why this is the case and its implications for public health, notably so if *G. duodenalis* is categorized as a zoonose which most experts agree that it looks to be (Feng & Xiao, 2011).

As mentioned previously, water sources are thought to be a main source of human infection with the parasite and Scottish Water supplies have previously been investigated for the presence of *G. duodenalis.* Smith *et al* (1993) found that both raw and final waters in some water supplies contained viable *G. duodenalis* cysts in Scotland. More concerning is that the highest level of cysts found was in a final water sample which was highlighted as a concern by the authors. This work is now somewhat out-dated and it is hoped that the situation has improved due to the advancement in water treatment methodologies which have occurred within the last 20 years since the work was published. In order to determine if this is the case and to investigate possible reductions in cyst numbers in both raw and final waters since 1993, it would be desirable to re-assess the situation in a more recent time period. To do this DNA

samples from a large amount of water sites around Scotland will be investigated during this project for the presence of *G. duodenalis* DNA using quantitative PCR techniques. Furthermore cattle faecal samples from a location near Edinburgh will also be investigated in the same way, as such animals are thought to be reservoirs for environmental contamination. Comparison of these sample sets could provide insight into assemblages most commonly found in the environment and thus contribute to our understanding of the threat of *G. duodenalis* assemblages to public health within Scotland.

1.3 Nomenclature history of *Giardia* species

Giardia as a species has had a highly debated and lengthy history with regards to its correct nomenclature. Since its first mention in 1681 the subject of *Giardia* nomenclature is still somewhat under debate today, greater than 300 years later which reflects the complexity of the parasite as a species. With extensive early work by pioneering authors such as Filice and Kunstler, academics have been able to agree on nomenclature of the species as a whole and differences of opinion are now focused on the correct nomenclature of sub-species, mainly due to dynamics of the different parasite sub-species and their epidemiology, both of which are still somewhat unclear.

First written documentation of *Giardia,* although unknowingly at the time, was noted by van Leeuwenhoek in 1681 upon investigating his own stools samples using a microscope. He also noted through repeated observations that the flagellated organism was present in these samples when they were diarrhoeic, however largely absent when well formed. Leeuwenhoek did not deduce that the organisms he had repeatedly observed (termed ‘animalcules’ by Leeuwenhoek in his writings) were responsible for the diarrhoea that he had been experiencing and instead deduced it had been his diet causing the symptoms (Dobell, 1920). Leeuwenhoek’s accurate description of the parasites general morphology, notably the presence of flagella and its shape, in combination with his symptoms, enabled it to be identified when combined with insight from other researchers in later years. In 1859 the organism was again observed by Vilém Dušan Lambl, a Czech physician who documented the parasite in greater detail than Leeuwenhoek could at his time. Lambl however miss-identified the organism believing it to be of the genus *Cercomonas,* as opposed to an unconfirmed and at that time, poorly, documented species of protozoan (Lambl, 1859: as cited in Adam, 2001.).

Years later, in 1875 and 1882, similar organisms were also noted in the intestines of both rabbits and tadpoles respectively. Davaine (1875) noted the presence of similarly structured organisms in rabbits and proceeded to name them *Hexamita duodenalis* due to the organism's flagellated structure which is shared with this genus, and him being unaware of the similarities between this organism and the one described by previous workers (as cited by Monis *et al,* 2009). Several years later, Kunstler (1882: as cited in Adam, 2001.) had found a similar unknown intestinal protozoa in the gut of tadpoles, now believed to be *Giardia agilis*, which he proceeded to term '*Giardia'* in honour of the Parisian Zoology Professor Alfred Mathieu Giard. This was the first illustration in which the nomenclature *Giardia* was used to describe the protozoan, and from this point it became the established correct identifier for the genus of protozoan in the scientific community. Lambl's error in nomenclature of the genus and species was not investigated until 1888 whereby it was corrected by Blanchard, who suggested the new name '*Lamblia intestinalis'* in homage to its first detailed documentation by Vilém Dušan Lambl (Blanchard, 1888 as reviewed in Monis *et al,* 2009). For years this process continued and there still remained a great deal of debate for the correct nomenclature of the protozoan, with various additional names being suggested as more information became available about the parasite. This lead to multiple names being used by different researchers for the same species of *Giardia,* that is, until the method was revolutionized half a decade later by Francis Filice (1952). Filice published a comprehensive morphological description of the genus allowing defined rules for species assignment. This was well received in the field as species had been assigned previously largely around host specificity. By this time the scientific community had largely accepted '*Giardia*' as the genus for the flagellated parasite and publication of the paper by Filice allowed placement of the organism into specific species dependant on specific structural similarities: *Giardia duodenalis*, *Giardia muris*, and *Giardia agilis.* The species list has since been confirmed using ribosomal RNA gene sequencing and has been backed up by the sequencing of other genes (Plutzer *et al*, 2010). Additional species have since been added to this list, named *Giardia ardea and Giardia psittaci* which have been found to infect birds and *Giarida microti* which infects rodents (Table 1). The different species are differentiated by observations made by light and electro-microscopy, ranging from shape of trophozoite, to differences in flagellae or ventral disk characteristics (Adam, 2001). As can be seen in Table 1, the majority of *Giardia* species have a specific host type, with *G. agilis* infecting amphibians, both *G.*

muris and *G. microti* infecting rodents and *G. ardeae* and *G. psittaci* both infecting birds. An additional species recently described by Upton & Zien (1997) has also been noted to infect reptiles, termed *G. varani*, however this species still remains to be confirmed genetically and so is still subjective (reviewed by Ryan & Cacciò, 2013) .

Table 1 - The Recognised species in the genus of 'Giardia'

Species Name	Host Species	Morphological Characteristics	Trophozoite Length	Trophozoite Width
G.duodenalis	Domestic and Wild Animals, including humans	Pear-shaped trophozoites with claw shaped median bodies.	12-15µm	6-8µm
G.agilis	Amphibians	Long, narrow trophozoites with club shaped median bodies.	20-30µm	4-5µm
G.muris	Rodents	Rounded trophozoites with small round median bodies.	9-12µm	5-7µm
G.ardeae	Birds	Rounded trophozoites with prominent 'notch' in ventral disk and rudimentary caudal flagellum. Median bodies round/ oval to claw shaped.	~10µm	~6.5µm
G.psittaci	Birds	Pear shaped trophozoites with no ventro-lateral flange. Claw shaped median bodies.	~14µm	~6µm
G.microti	Rodents	Trophozoites similar to G.duodenalis. Mature cysts contain fully differentiated trophozoites.	12-15µm	6-8µm

Table 1 - This table shows the recognised species of the Giardia genus as from 1952 based on the work by Filice (1952) and additional species identified since then. The work placed the different *Giardia* organisms into six distinct species based on their morphological features: *G.duodenalis, G.agilis, G.muris, G.ardeae, G.psittaci* and *G.microti.* Table adapted from Monis *et al* (2009).

The exception of this is the species *G. duodenalis (syn: G. lamblia or G. intestinals* dependant on views of nomenclature), which has a much wider degree of host specificity and has been found to include humans, domesticated and livestock animals, as well as many wild mammals (Feng & Xiao, 2011; Ballweber *et al*, 2010; Plutzer *et al*, 2010; Cacciò & Ryan, 2008; Thompson *et al*, 2000).

Despite the achievement of species differentiation in the wake of Filice's work, and further molecular confirmation of species, to this day there is still debate between academics on the correct nomenclature of the species which infects humans, resulting in either *Giardia duodenalis, Giardia lamblia* or *Giardia intestinalis* being used interchangeably to describe the same species (Monis *et al*, 2009). This issue is indeed undesirable as being unable to designate a specific accepted name to this species of *Giardia* adds further unwelcome confusion to the already complex area of work. *Giardia lamblia* is commonly seen to be used in literature often in terms of human derived *G. duodenalis*, notably from medical literature, along with inconsistencies in

publications focused on human giardiasis (Thompson *et al*, 2000). Thompson *et al* (2000) reiterated a point made in one of their previous papers (Thompson *et al*, 1990), that there is no taxonomic reasoning to use the name *Giardia lamblia* and so discourage its use. Meyer (1985), also as referenced by Thompson *et al* (2000), highlighted that when the species is referred to as '*Giardia lamblia'* it mistakenly suggests that the human derived *Giardia* is somehow unique compared to other forms, which is not the case. The names *G. intestinals* and *G. duodenalis* are also often used with varying frequency. This is however different to the debate on using the term *lamblia*, as both *intestinalis* and *duodenalis* are both technically acceptable dependant on differing perceptions of nomenclature rules in Zoology (Thompson *et al*, 1990 & Thompson *et al*, 2000). Throughout this work the species will be referred to as *Giardia duodenalis.* This name seems most appropriate from Filice's pioneering work on the field (Filice, 1952) until further, more advanced frameworks for identification of *Giardia* species are established there is no reason to deviate from this naming.

1.4 *Giardia duodenalis* – Assemblages and Sub-Assemblages

G. duodenalis is the only species of the *Giardia* genus which is known to infect humans in combination with a wide range of mammals, when compared to other known *Giardia* species. Extensive work has been carried out on *G. duodenalis* in recent years with the advent of increasingly effective molecular tools (Andrews *et al*, 1989; Monis *et al*, 2003; Thompson & Monis, 2004; Cacciò & Ryan, 2008), allowing in-depth insight into the species population structuring. As mentioned previously *G. duodenalis* has been

Table 2 - Known Host List of *Giardia duodenalis* Assemblages

G.duodenalis Assemblage	Host Species
A	Humans and primates, livestock animals, cats, dogs and some wildlife animal species
B	Humans and primates, cats, dogs and some wildlife animal species
C	Dogs and other canid species
D	Dogs and other canid species
E	Livestock animals
F	Cats
G	Rats
H	Marine species (range of pinnipeds)

Table 2 - A table highlighting the many assemblages of *Giardia duodenalis,* ranging from A - H and their respective host species (Table adapted from Ryan & Cacciò, 2013).

found to in fact consist of a cluster of eight genetically separate forms termed 'assemblages', ranging from 'assemblage A' to 'assemblage H', most of which have specific host requirements. These assemblages appear to have limited differences morphologically; however investigating protein or DNA polymorphisms can separate them into the different assemblages (Table 2) (Ryan & Cacciò, 2013).

Two key methods used for characterizing *G. duodenalis* assemblages are DNA based investigations and enzyme electrophoresis. Work using the latter was employed in early work in identifying the large heterogeneity seen in *G. duodenalis* genetics (Monis *et al*, 2009). *G. duodenalis* DNA based investigations are able to identify different assemblages of the species upon finding differences in sequences of specific genes.

Genes such as glutamate dehydrogenase (gdh), triosephosphate isomerase (tpi) and β-giardin (bg) are often used to establish differences between *G. duodenalis* assemblages. Phylogenetic analysis is also employed to ensure that the dissimilarity is not due to gene copy heterogeneity or intra-genotypic variations and following this, closely related genotypes of *G. duodenalis* were assigned into specific assemblages (as reviewed by Plutzer *et al*, 2010).

1.4.1 *Sub-Assemblages of Giardia duodenalis and the debate for further speciation.*

Using enzyme electrophoresis Mayrhofer *et al* (1995) were the first to establish that all human infective isolates of *G. duodenalis* were able to be assigned into two specific genetic assemblages, A and B. They found that isolates from humans were geneticcally distinct from both *Giardia muris* from rodents, and surprisingly, from the *G. duodenalis* isolates found in cats (assemblage F). The human derived isolates fell into two specific genetic assemblages, A + B, however these were extremely heterogeneous and the genetic difference between them in some cases was larger than those separating some bacterial genera, which is unusual.

As stated in a previous section, the existence of 'sub-assemblages' in both assemblages A + B has been proven, revealing clusters of genetically comparable isolates within the two assemblages. These 'sub-assemblages' have been referred to as 'AI' (A1), 'AII' (A2),'AIII' (A3) & 'AIV' (A4) within assemblage A and 'BI' (B1), 'BII' (B2), BIII' (B3) and 'BIV' (B4) within assemblage B (Thompson & Monis, 2011b; Monis *et al*, 2003; Cacciò *et al*, 2008) based on differences in protein polymorphisms revealing

genetic distance between each. Mayrhofer *et al* (1995) also showed that isolates belonging to assemblage A were much more heterogeneous when compared to isolates from assemblage B. Further work demonstrated that isolates of assemblage A + B are also known to have differing host specificities from one another, dependant on their sub-assemblage group, which further complicates an already complicated population structure.

Assemblage A subgroup AI is infective to a wide range of hosts including but not limited to humans, livestock and companion animals, however AII is restricted largely only to humans, whilst AIII is thought to be more limited to wild ruminants (Cacciò & Sprong, 2011c). The situation is similar for sub-assemblages in B, where BIII and BIV tend to infect humans compared to BI and BII which infect animals (Cacciò & Sprong, 2011d). Based upon years of work and the finding of sub-assemblages within *G. duodenalis* as a species, it is now widely accepted that the level of genetic distance separating assemblages of *G. duodenalis* is so large, that the species itself requires splitting into several new species (Thompson & Monis, 2004; Feng & Xiao, 2011; Andrews *et al*, 1998; Ryan & Cacciò, 2013; Jerlström-Hultqvist *et al*, 2010; Cacciò & Ryan, 2008; Monis *et al*, 2009). Table 3 below shows a list of new species names suggested by various authors.

The work proposes that assemblages A and B, which are known to cause infection in humans, should be named '*G. duodenalis*' and '*G. enterica*', respectively. *G. duodenalis* assemblages C/D should be combined into a single species, no doubt due to their similar host specificities (Table 2), termed '*G. canis*' whilst assemblage F which infects cats has been termed '*G. cati*'. The assemblage which has been commonly found to infect ruminants and hoofed livestock animals was suggested to be named '*G. bovis*'. The rodent specific assemblage of *G. duodenalis* has been termed '*G. simondi*' (Ryan & Cacciò, 2013).

1.5 The Morphology and Lifecycle of *Giardia duodenalis*

G. duodenalis is a complex organism and some of its biology still remains a mystery. Its lifecycle alternates between two specially adapted stages of parasite development termed the 'cyst' and 'trophozoite'. Cysts are adapted for survival out with of the host setting and are highly resistant to environmental factors. The trophozoite is adapted to life within the host, multiplying within the host and it cannot survive in the external environment. Both of these lifecycle stages are crucial for reproduction and survival, each having specific roles to play in the lifecycle of the parasite and employing interesting methods to do so. The parasite is also interesting as it lacks a golgi apparatus, peroxisomes or mitochondria; features which are found in the overwhelming majority of eukaryotes. Giardia is however an anaerobic organism, meaning that it does not require organelles such as mitochondria to survive through oxidative phosphorylation, required by most cells to create energy in the form of ATP (Adam, 2001).

Table 3 – A List of Suggested New Species Names for *Giardia* genus.

Species (Plus previous assemblage if applicable)	Host species
G. duodenalis (assemblage A)	Humans and other primates, companion animals, livestock and wild animals
G. enterica (assemblage B)	Humans and other primates, dogs and some species of wild mammals
G. agilis	Amphibians
G. muris	Rodents
G. psittaci	Birds
G. ardeae	Birds
G. microti	Rodents
G. canis (assemblage C/D)	Dogs and other canid species
G. cati (assemblage F)	Cats
G. bovis (assemblage E)	Cattle and other hoofed livestock animals
G. simondi (assemblage G)	Rats
Assemblage H	Pinnipeds

Table 3 – This list shows both recognised species of the *Giardia* genus along with suggested new species names currently known as the assemblages (A-G) of *G. duodenalis*. Currently assemblage H has had no individual species name suggested for it as less is known about it compared to the other assemblages. Table adapted from Ryan & Caccio (2013).

1.5.1 *Cyst Morphology*

The *G. duodenalis* cysts are ovoid and measures roughly 5µm by 7-10µm in diameter and is protected by a cyst wall of between 0.3-0.5µm thickness (Figure 1 & Appendix 5.4.1). The outer cyst wall is composed firstly of a layer which consists of a lattice of filaments roughly 7-20nm long, which create a strong defensive cover around the parasite protecting it from the environment. This is particularly useful against temperature fluctuations or chemical exposure when outside the host, as well as inside (i.e. stomach acid etc.). Inside of this thick outer wall there are two inner layer membranes (Adam, 2001). The contents of the cyst, beyond the wall, include four nuclei, axonemes which used to form flagella and fragments of the deconstructed ventral adhesive disk (Ankarklev *et al*, 2010).

Figure 1 - Morphology of a *Giardia duodenalis* cyst

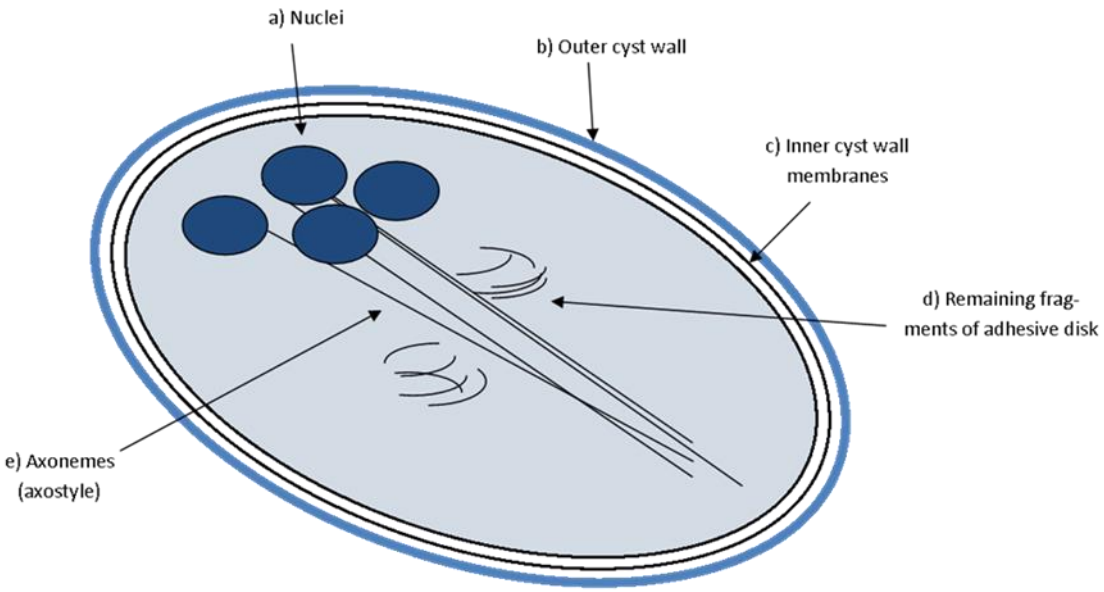


Figure 1 - This diagram shows the key morphological features identifiable within a mature *Giardia duodenalis* cyst. The significant features are those of: a) four nuclei within the anterior end of the cyst, b) a thick outer filamentous wall which provides the cyst with protection from the environment, c) a inner wall consisting of two membranes, d) a cluster of crescent bodies which once formed the adhesive disk of the trophozoite prior to encystation and e) flagellar axeonemes which can be seen spanning the longest axis of the *G.duodenalis* cyst (diagram based on information from Smith & Mank (2011f)).

1.5.2 *Trophozoite Morphology*

Trophozoites are tear-dropped shaped and measure approximately 12-15µm in length and 5-9µm in width. This parasite stage contains various features which help with its role in the life cycle (Figure 2). Trophozoites contain 4 sets (pairs) of cytoskeletal flagella, located ventrally, posteriorly, caudally and anteriorly which allow the protozoan to 'swim' in the internal host environment. These flagella exist within the trophozite for a short distance before exiting the parasite body and are then surrounded by a membranous layer upon projection from the cell itself. An adhesive disk on the ventral side of the parasite allows attachment to the host epithelial cells within the intestines and is an iconic feature of the species as a whole (Adam, 2001). These two features allow the parasite to resist peristaltic motions and remain inside of the small intestine for extended periods of time, allowing them to gain nutrition and divide within the host.

Figure 2 - Morphology of a *Giardia duodenalis* trophozoite

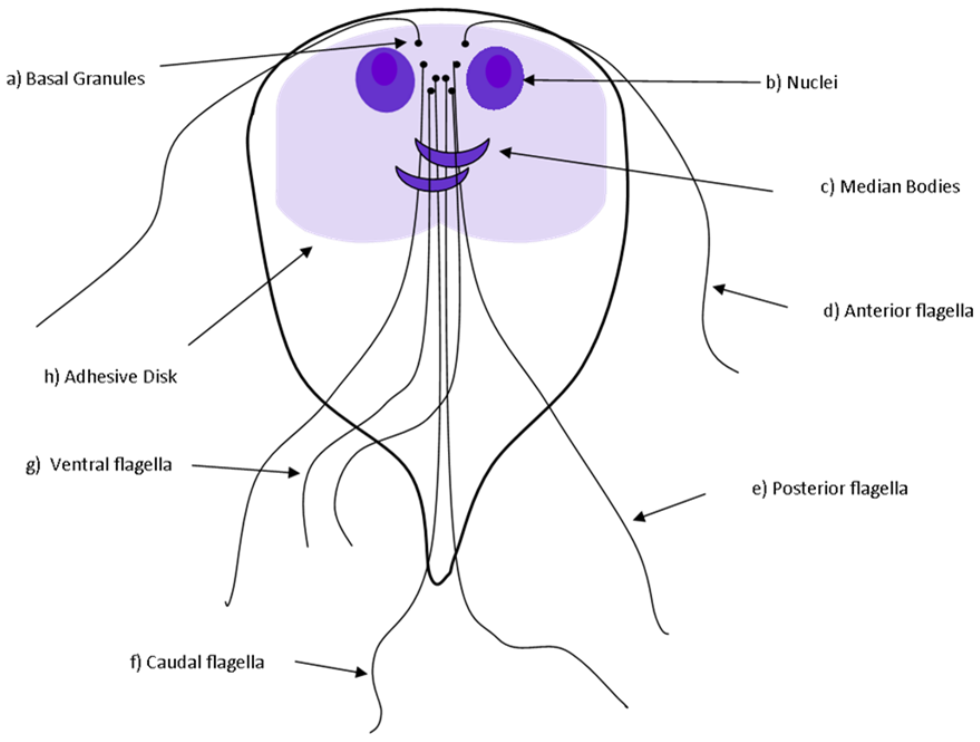


Figure 2 - This diagram shows the characteristic organelles of a *Giardia duodenalis* trophozoite. The parasite has 4 pairs of flagella which allow movement within the host environment which can be seen: d) anteriorly, e) posteriorly, f) caudally and g) ventrally. The trophozoite also contains two nuclei (b), basal granules (a) from which the flagella originate and an adhesive disk (h) located on its ventral side, which allows adhesion to the epithelial cells of the host intestine (Monis *et al*, 2009).

Median bodies which can be seen within the centre of the parasite are currently of unknown function (Ankarklev *et al*, 2010), however they are thought to be required for correct 'domed' formation of the parasites adhesive disk (Woessner & Dawson, 2012). Basal granules can be seen within the anterior end of the parasite close to the nuclei, from which the four pairs of flagella origionate, as seen within the trophozoite.

1.5.3 *Lifecycle of Giardia duodenalis*

The lifecycle of *G. duodenalis* consists of two main stages of the parasite as previously mentioned, termed 'cyst' and 'trophozoite' (Figure 3). Cysts are the environmentally resistant form of the parasite which contaminates the environment in vast quantities when shed in infected host faeces. Trophozoites are the form of the parasite required for reproduction and absorption of nutrients within the host. Upon excretion from the host the cysts are already infectious and have a low infectious dose (between 1-10 cysts causing infection). The cysts can remain infective for weeks to months in the environment, dependant on conditions, with higher or lower temperatures having detrimental effects on cyst viability (Cacciò *et al*, 2005). Infection begins in a host animal upon ingestion of these infective cysts (the parasite follows a faecal/oral lifecycle). The parasite passes through the low pH environment of the animal's stomach unharmed due to the protective cyst wall, allowing it access to progress into the intestinal tract (Ankarklev *et al.*, 2010). Once inside the upper section of the small intestine of the host the parasite replicative form begins to leave the cyst by a process termed 'excystation'. This process is known to be triggered by a combination of exposure to initially low pH conditions in the stomach, followed by exposure to contents of the small intestine (higher pH, low cholesterol and intestinal chemicals) and is a relatively quick process lasting only around 10 minutes (as reported by Buchel *et al*, 1987).

The process begins with cytoplasm retracting within the cyst, allowing the peritrophic space to enlarge and vesicles form on a pole end of the cyst between the internal contents of the cyst and the cyst wall. A hole in the cyst forms as the future trophozoite (termed excyzoite at this point as it does not resemble a trophozoite) within the cyst detaches and begins to emerge from the hole formed on the end of the cyst. Initially the excyzoite flagella emerge from this hole in the cyst, followed by the rest of the excyzoite leaving an empty husk behind. The emerging excyzoite inside the cyst contains two sets of two nuclei via previous karyokinesis during late encystation in cyst

maturity; however it only has the morphology resembling a single excyzoite/trophozoite during this time (single set of 8 flagella etc).

Figure 3 - The Lifecycle of *Giardia Duodenalis*

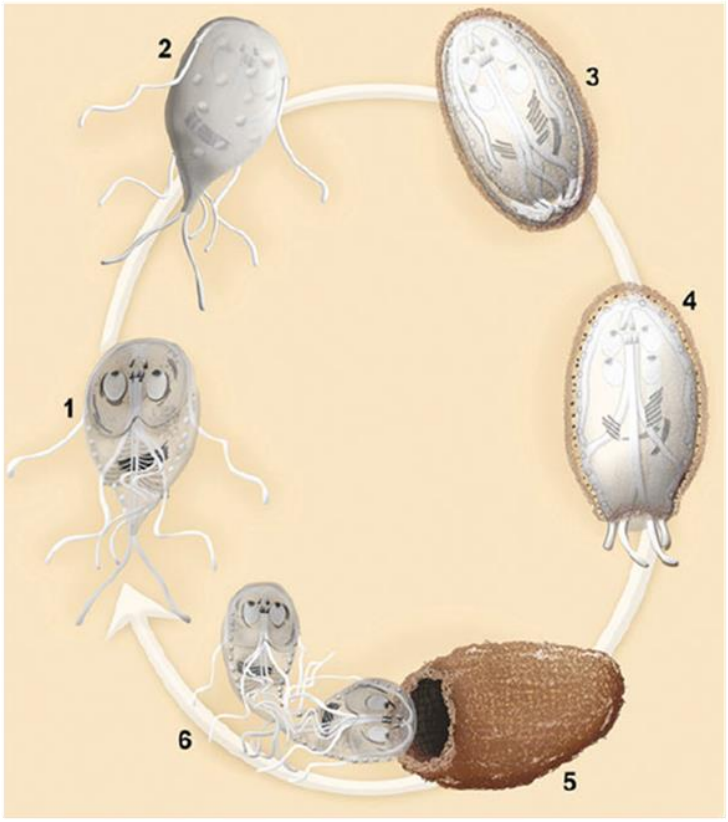


Figure 3 - This diagram shows the life stages and cycle of *Giardia duodenalis* within a host. 1) Trophozoites develop from a previously ingested parasite cyst and infect the intestinal epithelial cells of a host. 2) Trophozoite encystation begins due to chemical ques within the host environment when the parasite moves further down into the small/large intestines. Encystation-Specific Vesicles (ESV) can be seen appearing within the trophozoite. 3) Cyst wall forms from proteins released by the ESV which have now migrated to the surface of the parasite. Trophozoite structures retract and disassemble, with some fragments still being observable within the cyst. The two nuclei within (binucleated) each divide, resulting in a cyst containing four nuclei. Cyst is now mature and is released into the environment in host faeces. 4) The cyst is ingested by a new host via contaminated food or water. Encystation begins with a hole appearing in a pole of the cyst and flagella of a new excyzoite begin to exit the cyst. Enzymes appear via new vesicles and assist in breaking down the cyst wall. 5) Parasite fully leaves the cyst leaving an empty shell behind. 6) The new excyzoite beings to divide and forms two binucleated trophozoites by cytokinesis. Trophozoites adhere to the intestinal epithelium for nutrition and cause a variety of problems for the host, before moving further down the digestive system and beginning to encyst. Information and diagram taken from (Benchimol & De Souza, 2011g).

Following this emergence of the excyzoite from the cyst wall cytokinesis begins within the cell, resulting in two binucleated trophozoites appearing from a single parent excyzoite. These will then both divide asexually by binary fission to create additional trophozoites, multiplying the amount of parasite forms exponentially within the host. Binary fission of trophozoites is known to occur in three stages (Tumova *et al* (2007),

as reviewed by Dawson *et al* (2011) during which the parasite alternates between being adhered to epithelial cells of the intestine and being free swimming. The trophozoites adhere to the intestinal epithelial cells using adhesive disk located on their ventral end and eventually colonize the surface of the intestine surface area, which is what causes a variety of problems (symptoms) for the host animal. When these trophozoites move further into the small intestinal tract biliary excretions and environmental cues cause them to begin a process called encystation, which results in the infective and environmentally resistant cyst lifecycle stage of the parasite being formed from existing trophozoites.

The entirety of what causes the trophozoite to encyst is currently not fully understood, however it is believed that there are a variety of chemical in factors at play in the process. The initial stages of encystation involve the trophozoite beginning to change shape, internalizing and disassembling key morphological features such as the adhesive disk and flagella. During this, the production of a cyst wall begins with encystation-specific vesicles appearing within the trophozoite, which migrate to the surface and release a protein required to create a cyst wall. During these stages of transformation the parasite is referred to as an 'encyzoite' as it does not resemble a trophozoite. During late stage encystation the nuclei both divide, leading to the presence of two sets of two nuclei within the newly formed cyst.

The cyst wall matures giving the parasite significant protection from environmental factors once completely transformed from its previous trophozoite stage. The cyst is then excreted by the host in faeces along with an amount of trophozoites which were unable to remain within the host's gut. These trophozoites will disintegrate after a short time as they are unable to survive outside of the host system and so do not contaminate the environment, however the cysts do. Once ingested by a new host the parasite cyst will restart its lifecycle by manoeuvring through the host digestive system until it reaches the small intestine, excysting and releasing trophozoites to initiate a new infection.

It has long been (perhaps wrongly) assumed that reproduction within *Giardia* species are asexual, with isolates remaining genetically identical throughout the lifecycle of the parasite (Adam, 2001). This has however recently been called into question with authors promoting the idea of possibility of genetic exchange between lineages of *G. duodenalis*, often with evidence to support their predictions. Currently there is still no

clear answer to the question of sexual reproduction in the parasite. It does appear from the evidence that there is a degree of genetic exchange occurring, however confirming and understanding how this happens in *Giardia* is still out of reach (Cooper *et al*, 2007; Cacciò & Ryan, 2008; Birky, 2010).

1.6 Filtration Methodologies for Waterborne Pathogens

As *G. duodenalis* can be transmitted by drinking contaminated water, effective filtration and identification of the parasite from water samples becomes a significantly important preventative measure when attempting to prevent outbreaks. Water companies throughout the UK, as well as most developed countries, have a duty to ensure that their water is safe for human consumption and filtered to ensure the absence of disease causing organisms. Water companies within the UK take regular samples of high risk areas to determine parasite load within a reservoir of water for human consumption. Although there are no legal laws for *G. duodenalis* levels within water, these do exist for other protozoan species notably *Cryptosporidium*. A review of waterborne disease outbreaks between the years of 2004-2010 reported that *G. duodenalis* accounted for 70/199 reported outbreaks of human disease due to waterborne protozoa within the developed world (Baldursson & Karanis, 2011). In the USA it is however monitored via the EPA Safe Drinking Water Act (SDWA) and regulated using US EPA Method 1623 (Method 1623.1, 2012), something which has had a positive impact on reducing cases of giardiasis within the country. Filtration methods are still currently at a level whereby 100% recovery of parasite numbers within samples is not achievable; however recoveries of between 41-70% are to be expected using the Filta-Max system (UK Environment Agency, 2010). There are currently no shortages of methodologies for water filtration techniques as can be seen in a paper by Wohlsen *et al* (2004). This means that the adaptation/improvement of existing systems, such as the Filta-Max system, is desirable compared to a complete overhaul of existing methods. Adapting and improving filtration techniques commonly in use is favourable as it reduces time required to implement the new method as well as limits the costs involved, which is important when implementing filtration systems throughout a country. Currently the Filta-max system relies heavily on manually operated wash station, which entails both a degree of repetitiveness as well as labour intensive steps. The nature of the methodology currently means that a single analyst preparing samples needs to be careful not to develop repetitive strain related injuries by processing too many samples over time. The

manual plunging of the wash station combined with manual rubbing of the membrane sample create strain on the arms and hands respectively when carried out properly to ensure a good recovery from the sample (see UK Environment Agency (2010) for details about this procedure). Furthermore to this, human error is always a factor when a process is carried out by an operator, something which a machine programmed correctly would not be capable of. Full automation of sample processing in water laboratories will no doubt be a possibility in the future and this is something which should be looked at in order to standardize processes more efficiently. The implementation of megasonic waves onto the existing filtration method has been carried out by Kerrouche *et al* (2015) for the waterborne parasite *Cryptosporidium* and is a key step in this automation process. The method reduced the manual labour involved with the existing methodology whilst still keeping recovery levels at an acceptable level by substituting manual steps for semi-automated ones. Notable improvements were a simplification of the sponge filter and membrane wash steps as well as allowing a time consuming centrifugation step to be skipped, which was highlighted by the authors as the main step which hindered the automation potential of the existing process. The incorporation of this megasonic methodology into *G. duodenalis* elution could potentially also have the same benefits as were found for *Cryptosporidium*, warranting further investigation. Additionally it is understood that *G. duodenalis* is a less robust parasite compared to hardier species such as *Cryptosporidium* and so we propose that additional, gentler, methods of elution (such as megasonic method) for the species may be useful to improve current systems. If found useful for *Cryptosporidium* as well as *Giardia* species its implementation could also be useful in combining these two pathogens into a single test, as currently these must be carried out separately due to unreliable recoveries when processed together (see DWI Report 70-2-155 (2003) for more information).

1.7 Zoonotic giardiasis – Risk or no risk?

Whether *G. duodenalis* is a zoonotic organism is still a hotly debated topic between authors which gains interest with each year worldwide. More and more studies are now being carried out in this area in an attempt to understand the true story of zoonotic *G. duodenalis*, however often the studies contradict each other, somewhat furthering unknowns. As the human infective forms (assemblages A + B) of the parasite can have the ability to infect both humans and animals, often indiscriminately, they are deemed

by many authors to be a zoonotic organism (Feng & Xiao, 2011). There is now a range of evidence to both support and deny this claim, which makes the true picture difficult to discern due to data sets which appear to contradict each other. A comprehensive review by Feng & Xiao (2011) details a wide range of evidence to support the claims for and against *G. duodenalis* being classed as a zoonose. It is thought that assemblage A is the most likely to be at least somewhat zoonotic due to a host range which infects a wider range of hosts than B, including livestock (Ryan *et al*, 2013). However, assemblage B has also however been observed in a range of animals as well as humans, which still suggests potential for zoonotic crossovers to humans even if to a lesser extent than assemblage A (Table 3). The assemblages thought to be most of threat to humans (A and B) also require further typing beyond simply identification of assemblage present. Sub-types within both assemblages act in different ways, with some being able to infect humans, and others only infective to animals. It is thought that the human infective assemblages appear to have independent cycles of infection due to their numerous host species, allowing lifecycles within specific reservoirs to carry on for generations before entering human hosts possibly through contaminated water or food for human consumption (Hunter & Thompson, 2005; Monis *et al*, 2009). It is however largely unknown how these independent lifecycles interact with each other and this must be understood in detail before conclusions can be drawn from this. Waters *et al* (2016) have found, by using mathematic models, that even if the infection cycles do not cross, there is still a high potential for human infection via infected animals, highlighting this as an issue requiring definite attention.

Notably farmyard animals, companion animals and wildlife throughout the world have all been suggested to pose a risk for zoonotic *Giardia* infection.

1.7.1 *Livestock and Farmyard Animals as Risk of Zoonotic Infection*

In the past countless studies have found that the majority of livestock animals worldwide are infected with *G. duodenalis* assemblage E (as reviewed by Ryan *et al*, 2013; Feng & Xiao, 2011), which would be expected due to the assemblage having only livestock animals as hosts. This assemblage is however not the only assemblage which can infect livestock, with the potentially zoonotic assemblage A also being present in some animals. It has also been found that in some cases Giardia assemblages A + E can also co-infect a host (Geurden *et al,* 2012). Farm animals and environments are often suggested to be a potential reservoir for human infective *G. duodenalis* assemblage A

due to both the large numbers of livestock animals present globally and the potential of animals to create widespread contamination due to waste produced, potentially containing infective cysts (Ryan *et al*, 2013). The potential of these animals contaminating the environment is however thought to vary dependant on the practices of the farm and region of the world. An example for a farm practice that allows parasite dissemination is to allow cattle direct access to a river or steam, which will allow them to defecate directly into it, potentially spreading infective cysts downstream to other environments (Budu-Amoako *et al*, 2011). This can be prevented by educating farmers, however it is difficult to enforce in practice.

Cattle are suggested to be a large reservoir of the potentially zoonotic assemblage A and are the focus of numerous publications (O'Handley *et al*, 1999; Appelbee *et al*, 2003; Ralston *et al*, 2003; O'Handley *et al*, 2000; Trout *et al*, 2007; Hoar *et al*, 2009; Santin *et al*, 2009; Sprong *et al*, 2009; Miguella *et al*, 2012; Ryan *et al*, 2013). Sprong *et al* (2009) found that in the majority of cattle samples investigated (total 562), 47% of these were infected with assemblage E within Europe. This assemblage of *G. duodenalis* appears to be widespread and the current consensus is that it is the most commonly observed assemblage in cattle worldwide. More recently however a similar study by Geurden *et al* (2012) found that in a sample size of 2072 cattle from areas of Europe, when infected with *Giardia* (~45% or ~1000 animals within study), on average 43% of them were infected with *G. duodenalis* assemblage A. This would suggest that the majority of cattle within Europe are not infected with the potentially zoonotic assemblage, however a relatively large amount of them still have a zoonotic potential. This has also been suggested previously by Feng & Xiao (2011), who described that more animals may be infected with assemblage A than realised. Even with a small minority of cattle being infected with assemblage A, this number cannot be overlooked as the potential for zoonotic transmission to humans is possible. Interestingly within this study there were differences in positive animals identified within samples from different countries, showing that within each country assemblages could be spread in varying quantities. Within the work by Geurden *et al* (2012) assemblage A prevalence in *Giardia* infected cattle was found to be 61% in France and 41% for Germany, however only 29% within the UK and 28% in Italy. Similar reports to this have been published from areas all around the world, showing that either assemblage A or assemblage E is more or less evident in cattle populations' dependant on geography (Uehlinger *et al*, 2006; Santín *et al*, 2009; Feng & Xiao, 2011). Assemblage B has been identified within

some cattle populations in China and also in Canada (Uehlinger *et al*, 2006; Coklin *et al*, 2007; Dixon, *et al*, 2011; Liu *et al*, 2012), which is a less common situation when compared to other areas of the world such as Europe. Notably Canadian work has shown that dairy cattle appear to be more commonly infected with potentially human infective assemblages, raising their threat for zoonotic transmission over beef cattle. Several longitudinal studies as reviewed by Feng & Xiao (2011) who have found that cumulatively the infection rates within cattle range between 73-100% infection with *Giardia* species, however clearly assemblage prevalence varies significantly dependant on location.

Despite previous information pointing towards assemblage A being present in cattle, it is however unknown if these infected animals are to blame when human cases of infection arise. A smaller number of studies have been carried out to actual link these factors, which are paramount when attempting to understand the infection dynamics within populations (Khan *et al*, 2011; Ehsan *et al*, 2015; Sprong *et al*, 2009). The problem is further complicated by the fact that *G. duodenalis* assemblage A consists of a number of distinct sub-assemblages which each infect specific hosts. Of these sub-assemblages (AI, AII, AIII and AIV) only AII is thought to be the most serious threat of infection in humans (Xiao & Fayer, 2008). Most cattle tend to be infected with AI (at least within Europe) (Sprong *et al*, 2009) however a large number of studies did not confirm the specific sub-assemblage of assemblage A infecting an animal, leaving the zoonotic threat levels somewhat unconfirmed for assemblage A. Sub-assemblage AIII tends to be more restricted to wild animals as opposed to livestock; it is however occasionally found in livestock (Cacciò & Sprong, 2011c).

In cases where the sub-assemblage of the infection has been described, results are contradictory. A case-control study by Khan *et al* (2011) found that dairy farm workers in India were infected with a sub-assemblage of A, AI, which suggested that the disease was contracted from contact with the cattle due to its frequency in the farm cattle. As previously mentioned however this is the sub-assemblage thought to be less infectious for humans. This is inconsistent with a study in Bangladesh by Ehsan *et al* (2015) in which there was no link between cattle and the population rearing them in the area. In the Bangladesh study the majority of cattle were infected with assemblage E, whilst the human samples contained human specific sub-assemblages of assemblages A and B. This would suggest that the two infection cycles within cattle and humans in this area were not linked and were functioning independently. Work in Ontario, Canada, by

Dixon *et al* (2011) however came to the conclusion that within dairy cattle, more so than beef, there was a potential zoonotic risk of *Giardia* infection for humans.

Outbreaks believed to be caused by cattle directly have been suggested by Olson *et al* (2004) as being most likely caused by humans as opposed to cattle. Undoubtable is that the cattle could have contaminated the surrounding area, particularly surface waters which spread the cysts, however these animals may have been infected due to human activities previously. Olson *et al* (2004) also mentioned that the infection is difficult to be removed once it is in the cattle population by widespread disinfection and treatment of herds, causing a cycle of re-infection for adult and young cattle alike once introduced by human activity.

The situation is much the same in other livestock/farmyard animals, such as sheep, goats and pigs. These animals are most commonly infected with *G. duodenalis* assemblage E and infrequently with A (Robertson, 2009). Work by Sprong *et al* (2009) found a similar situation within goats as in cattle, where the majority was infected with assemblage A. Most goats within the study were shedding assemblage AI (78% of the isolates typed) and the remaining samples (22%) typed as AII. A review of the zoonotic giardiasis threat from sheep by Lucy Robertson (2009) detailed that the majority of sheep were infected with assemblage E, however via extrapolated data from numerous studies she estimated that around 30% of isolates from sheep have the potential to be zoonotic. Pigs have also been found to contain a similar range of assemblages to cattle, sheep and goats, with assemblage E being dominant. Pigs have also been observed to contain a small number of sub-assemblage AI isolates, again similar to other farmyard animals (Feng & Xiao, 2011). Less common farmyard animals have also been found to be infected, such as Alpaca, however the data on these animal varies with authors suggesting increased or decreased assemblage A and E isolates in different studies (Cebra *et al*, 2003; Trout *et al*, 2008; Gomez-Couso *et al*, 2012).

A suggestion by Feng & Xiao (2011) into the lack of human infective *G. duodenalis* isolates being detected in livestock compared to species specific assemblages is that competition within hosts, which are susceptible to both human-infective assemblages (A) as well as host-specific assemblages (E), may prevent the human infective assemblage becoming the dominant parasite. This would lead to a decrease of human infective assemblages in species in which co-infection with a host specific assemblage of *G. duodenalis* occurs. This is often seen to be the case, with assemblage E being

much more common compared to assemblage A in cattle, reviewed by Feng & Xiao (2011) and by Olson *et al*, (2004). The hypothesis is not unrealistic as competition between isolates is proven to occur in *in-vitro* cultures (Thompson *et al*, 1996) and similar situations have been reported for similar protozoan parasites (Mideo, 2009).

1.7.2 *Companion Animals and Risk of Zoonotic Infection*

Companion animals have also been thought to be a potential source of infection for human infective assemblages of *G. duodenalis*, notably cats and dogs (in which there are 77 million and 93 million in the USA alone (Esch & Petersen (2012)). A comprehensive review of the status of zoonotic *G. duodenalis* in these two common house hold pets was carried out by Palmer *et al* (2008). Their study in Australia found that the zoonotic risk of *Giardia* infection of these animals appeared to be low for humans. When infection was found in a companion animal, it was suspected that potentially it could reflect the practices of human activity in the area. Thompson (1999) found that in urban areas dogs were just as likely to be infected with assemblage A as they were with the dog specific assemblage C, and it was speculated that this was due to low infection pressure. This would suggest that whatever assemblage the dog was exposed to, the dog will become infected with. This also suggests that the zoonotic potential for *G. duodenalis* was low in this case; however the opposite was found in a small community in India (as reviewed by Palmer *et al*, 2008) where dogs and owners were both found to be infected with assemblage A of the parasite. This was however suggested to be due to the practices of the humans in the area and the availability of human faeces for dogs to consume in the community. It is more than likely however in this population that the parasite is zoonotic as the parasite is readily being passed from human to animal, undoubtedly due to poor hygiene. Results and findings like these reinforce the fact that zoonotic giardiasis is due to a variety of factors, however a cause certainly appears to be the involvement of humans within an area.

Humans have a high potential to introduce the pathogen into the environment in which it can spread, surviving in various hosts due to wide host ranges. As a result, the parasite may only come into contact with humans again occasionally as it can survive for extended periods of time without requiring a human host. Feng & Xiao (2011) reviewed the status of companion animals with regards to zoonotic infection with *G. duodenalis*. Their findings, based on a large number of studies, show that when human

contact is involved (i.e dogs owned by humans etc) there is an increased likelihood of *G. duodenalis* assemblage A being present in the animals. This will increase the levels of human infection in the population as a concequence. Feng & Xiao (2011) also noted that there is another infection cycle from dog to dog, passing assemblage C + D to each other, resulting in two potential separate cycles within the canine population. Thompson (2000) has also suggested that, like in cattle with assemblage E, competition may occur in the dog specific assemblages, C and D, resulting in suppressed assemblage A infection.

Cats are thought to be less often infected with assemblage A as a whole, with the majority being infected with assemblage F – the cat specific assemblage. Some cases have however been reported with cats being infected with assemblage B (Palmer, 2008). Cats and dogs are very different animals; dogs generally come into contact much more frequently with humans due to behavioural habits which could enable transmission of the parasite from dog to human to be much more frequent (however rare access for dogs to human faeces will limit the infection being passed back to the dogs, especailly in developed countries). Studies into giardiasis in dogs and cats are far and few between and often differ in results, similar to other animals. A large scale analysis by Bouzid *et al* (2015), investigating work which used canine and feline stool samples, found that prevalence rates varied, with dogs and cats at 15.2% and 12% respectively when comparing studies worldwide. This study highlighted that there was a difference in methods used and that these affected results, suggesting standardization within the community is desirable to better identify the real parasite epidemiology within the animals. Ballweber *et al* (2010) also found that when reviewing a range of papers much of the evidence suggested that there is no zoonotic potential; however some papers did show evidence of it being possible in some communities. They also highlighted the lack of data for *Giardia* in dogs and cats within industrialized nations, which is in need of addressing.

1.7.3 *Wildlife and Risk of Zoonotic Infection*

It is known that both *G. duodenalis* assemblage A and B infect wild animals as well as humans and domestic/farmyard animals. Feng & Xiao (2011) noted that beavers are often a suggested source of potential water source contamination where they are found. They were also the reasoning behind the World Health Organisation (WHO) classifying giardiasis as a potentially zoonotic disease (Thompson, 2004). The disease is commonly

called ‘Beaver fever’ due to the past association with the rodents and disease presence in watersheds. Interestingly however the consensus as found by Feng & Xiao (2011) appears to be that it is very much unknown whether the beavers introduce the parasite to the watershed, or have simply become infected by *Giardia* already present in the supply. Regardless, this amplifies levels of parasite within the catchment. Beavers have previously been seen to be infected with both assemblage A and B of the parasite (Sulaiman *et al*, 2003; Fayer *et al*, 2006; Feng & Xiao, 2011). Non-human primates are known to be infected with both assemblages A and B and therefore where found, they could be involved in potential zoonotic contamination of the environment which could lead to human cases (Graczyk *et al*, 2002; Levecke *et al*, 2007; Volotao *et al*, 2008, Ye *et al*, 2012). A range of wild animals have been identified to contain assemblages A + B (Sprong *et al*, 2009). Reviews of the area by Ryan & Cacciò (2013) and Feng & Xiao (2011) reported that non-human primates have been found to contain isolates belonging mostly to assemblage B, wild ungulates tend to be predominantly shedding assemblage A as opposed to assemblage E, as expected. Wild carnivores appear to be infected with a mix of assemblage isolates, including A, B, C and D. The assemblage infecting them appears to vary with geography (African hunting dogs were found mostly not to shed assemblages C or D, which is again unexpected). Marsupials form a large amount of the native wildlife in Australia and these animals were found to contain mainly isolates of assemblage A and B, much like other reported wildlife worldwide. Marine mammals such as seals were found to be infected with assemblages including A, B, D and H, dependant on host species (most dolphins and porpoises contained assemblage A isolates whereas seals were more mixed between assemblages A, B, D and H). When infected with assemblage A, marine species were found to contain often either assemblage AI or AII. Shorebirds close to these ecosystems were also found to be infected with similar assemblage mixes, consisting of A, B or H. From the information from the reviews it is apparent that a large reservoir of human infective *G. duodenalis* exists within many groups of wild animals, which are often infected with assemblage A and B. Subtyping is desirable in these animals as a large proportion of ungulates will be infected with assemblage A subtype AIII or BI etc., which are non-zoonotic, however others will be infected with the human infective sub-assemblages, AII, BIII and BIV.

1.7.4 *Level of Threat of Zoonotic Giardia Assemblages to Public Health*

It would seem apparent that based on the evidence from a range of studies carried out, many as mentioned above, giardiasis is indeed a zoonotic disease. However the

prevalence of this zoonotic status should not be overstated and must be assessed on a case by case basis to fully understand the local risk. Geurden *et al* (2012) exemplified this well, showing infection differences within Europe. Much evidence previously found shows that the cycle of infection between humans and livestock (Dixon *et al*, 2011; Khan *et al*, 2011;), humans and companion animals (Palmer *et al*, 2008) and humans and wildlife (Sprong *et al*, 2009) have the ability to cross, resulting in human cases. Where this has been found it appears that the infection status is however local only, as other studies with similar hosts being investigated (i.e. both animals and humans) often disagree with findings (Ehsan *et al*, 2015). With recent advancements in understanding of *G. duodenalis,* the question now is not 'is *G. duodenalis* zoonotic', but more so 'when is *G. duodenalis* zoonotic'. Furthermore, it is now important to sub-type *G. duodenalis* assemblages A + B upon their identification in a population, as opposed to simply identification of assemblage. In doing this zoonotic potential can be addressed. With the advent of genetic tools this is now easier to do than ever before, meaning that future work where feasible should always aim for this information as a gold standard for inclusion in data. Increased understanding of specific sub-assemblage prevalence in both animal types and geographical areas will accelerate understanding of *G. duodenalis*, in both its epidemiology and risk to public health.

1.8 Infection, Diagnosis and Treatment of *Giardia duodenalis* in Humans

1.8.1 *Giardiasis and Infection in Humans*

G. duodenalis infection prevalence within the global human population varies between the developed and developing world, with infection estimates of between ~5% and ~20%, respectively (Roxstrom-Lindquist *et al*, 2006). Within the developed world strict control and filtration of drinking water, as well as high quality sanitation systems, prevent the disease being as prevalent as it is in poorer areas of the world. These factors make the disease a particular problem in less developed countries in which poverty within the population is rife. Despite this, outbreak events of the disease do occur within the developed world. Outbreaks are often linked to contaminated food and water sources, contaminated public swimming pools or children day care centres, causing spread of disease (Robertson, 1996; Nygard *et al*, 2006: Smith *et al*, 2006; Daly *et al*, 2010; Rimhanen-Finne *et al*, 2010). In 2006, giardiasis was added to the neglected disease initiative for this reason due to both its massively under reported status and effects on poverty stricken countries, as well as in developed countries (Savioli *et al*, 2006). Within developing countries low sanitation and healthcare availability allow the disease to spread rapidly within communities, leading to malnourishment and loss of life quality, not to mention productivity. Infected people within these poorer countries are debilitated by the symptoms, meaning that they may struggle to work, become malnourished and weakened, and thus descend further into the disease and poverty cycle.

A combination of factors makes giardiasis in humans both an under-reported and neglected disease in humans. The long incubation period makes it often difficult to determine the point of infection for the individual - unless there is an outbreak or obvious source in which the route of infection can be identified easily. Furthermore, diagnosis of the disease by professionals (mostly relevant to developed countries) requires some knowledge of possible causes of the symptoms, which are often miss-diagnosed due to their commonality with other diseases. Infection results in a wide array of symptoms from bloating, flatulence, steatorrhea and diarrhoea to malaise, weight loss and malabsorption. In certain cases hospitalization may be required, particularly in vulnerable individuals such as young children or the immunocompromised (Alexander *et al*, 2014).

Infection with *G. duodenalis* is known to have peaks within a population dependant on age group. The majority of cases of reported *G. duodenalis* infection

within the UK are within the age group of 0-4 years old with children more often at risk of infection than adults. The majority of cases within developed parts of the world tend to follow this suit (Cacciò & Sprong, 2011). This is however open to some bias as mothers with children exhibiting symptoms of diarrhoea will be more willing to bring their child to a GP for diagnosis, compared to an adult experiencing the same symptoms (short term diarrhoea, for example which is a common symptom). This will result in increased reported cases for this age group and could somewhat explain the high levels of children diagnosed with *G. duodenalis* infection, as opposed to an inherent susceptibility to exposure. Children are however known to be more susceptible to disease due to various reasons such as immune system developmental status, often resulting in disease outbreaks within nurseries and day care centres. Infection of the mothers of these children potentially accounts for an additional peak in adults observed between 25-44 (Savioli *et al*., 2006), however this is debateable and could be due to other factors, as in Scotland in 1996 a study found that mothers were not significantly more likely to become infected (Robertson, 1996). Increased exposure to increasingly likely infected children would however make sense as a factor for this age group's infection peak, perhaps setting a pre-disposition for exposure to cysts. Due to a mother changing a child's nappy numerous times in a day, an increase in exposure to infection would be expected in this age group.

Travel is also known to be a factor of infection within the UK and indeed the world, whereby adults (particularly young adults) travelling to areas with poor sanitation (E.g. eastern countries, areas of Africa and Asia) contract the infection before returning to their home country (Pollock *et al*, 2005; Alexander *et al*, 2014). Due to the fairly long incubation period, disease symptoms may not begin until they return home (Ekdahl & Andersson, 2005). *G. duodenalis* incubation periods vary within people, however the general accepted incubation period is of between 1-2 weeks from point of ingestion of cysts. Symptoms commonly last between 2-4 weeks (acute) however in some cases asymptomatic infection has been described; with the individual unaware they are infected which may last for a extended time (Gardner & Hill, 2001).

Furthermore to this, long-term (chronic) infection can occur, causing continuing issues for patients which can last for several months, if not longer, as previously mentioned (Katz *et al*, 2006; Robertson *et al*, 2006; Hanevik *et al*, 2007; Alexander *et al*, 2014). The disease is well known to affect people in different ways, with some being mildly (acute) by the disease and others heavily affected (chronic), or not affected at all

(asymptomatic). Chronic infection is thought to occur due to a complex range of factors from host and parasite, causing long term sickness and repeating symptoms and much suffering for the host. Host factors such as age, diet, immune status and even gut fauna are thought to be involved with the development of chronic infection. From the parasite, the ability to replicate and evade the host immune response is speculated to be involved in the process (as reviewed by Robertson *et al*, 2010; Solaymani-Mohammadi & Singer, 2010; Halliez & Buret, 2013; Bartelt & Sartor, 2015).

1.8.2 *Diagnostic Methods for G. duodenalis Infection*

A variety of methods exist when attempting to make a diagnoses on a patient infected with *G. duodenalis*, each have benefits and often a combinative approach is required (Gardner & Hill, 2001; Johnston *et al, 2003*). Symptoms of the disease vary considerably between patients and often many are generic of gastrointestinal disease (diarrhoea, stomach cramps, nausea etc.), meaning giardiasis cannot be immediately suspected by a general practitioner as the cause of illness in a giardiasis case. Most parasitic infections with an oral/faecal lifecycle are diagnosed based on the positive identification of parasite stages within the faeces of the infected person. This is no different for giardiasis and identification of *G. duodenalis* cysts within the stool of an infected person allows diagnosis of disease. The trophozoite stage is also passed in faeces, however as it is adapted to life within the host gut it doesn't survive long and breaks down quickly, becoming unrecognisable (Koehler *et al*, 2014).

An ova and parasite exam is a method of diagnosis which is carried out on between one and three stool samples of the potentially infected individual to attempt a diagnosis. A single stool sample can diagnose giardiasis in patients around 60-80% of the time; however the level of success increases with additional stool samples being tested. Three samples readily achieve diagnosis in over 90% of suspected cases; however *G. duodenalis* is known to have sporadic shedding cycles within hosts (Goka *et al*, 1990; as reviewed by Johnson *et al*, 2003). As such, there may be days when infected hosts faeces contains decreased numbers of cysts, compared with others when cysts are much more prevalent in a sample. This feature of *G. duodenalis* infection means that it is not unheard of to have greater than three stool samples taken for analysis over a period of time before a diagnosis is made. Additionally this process takes significant time as the sample must be taken, transported to, prepared, stained (where required) and viewed by a trained microscopy analyst in a public health

laboratory to enable a positive identification. This means that time taken, cost, labour and requirement of expertise on the microscope as well as human error are issues for the testing process. (Van den Bossche *et al*, 2015).

Particularly problematic cases may require more invasive endoscopic techniques to be employed if *G. duodenalis* infection is suspected, but cannot be confirmed by faecal tests. In these cases duodenal fluid biopsy or collection can be employed in order to identify trophozoite stages (microscopically) of the parasite active inside the host gut and thus confirm infection (as reviewed by Gardner & Hill, 2001). Various immunoassays have been, and still are being, developed which allow rapid identification of *G. duodenalis* cysts within a sample, potentially much more accurately, timely and objectively than standard microscopy techniques (Weitzel *et al*, 2006; Schuurman *et al*, 2007; Gaafar, 2011; Alexander *et al*, 2013; Koehler *et al*, 2014; Stark *et al*, 2014; Van den Bossche *et al*, 2015).

Antigen detection in faeces using enzyme-linked immunosorbent assays (EIA), non-enzymatic immunoassays, or staining of faeces samples using monoclonal antibodies are all employed for use in *Giardia* diagnosis and are often more accurate and more readily employed by public health bodies compared to simple microscopy. These advancements mean that the focus in diagnosis of giardiasis has moved from traditional microscopy and now tends towards confirmation of infection in samples using advanced machines. This movement is advantageous to public health for various reasons: 1) large numbers of tests can be carried out by machines simultaneously, as opposed to being limited by microscopic analysis time 2) tests are often objective with clear yes/no criteria for infection status of sample, 3) tests are often quantitative, which can reflect estimated presence of parasite numbers within a sample, 4) tests can be carried out relatively quickly, allowing faster diagnosis and thus faster patient treatment, 5) tests do not require large amount of human input, which leads to less human error involved.

It should be noted however that these tests do also occasionally have false positive/false negative results, which must be further confirmed where data looks suspect. In situations like these, a back-up of microscopy techniques can still be very useful in the confirmation of infection if required. Van Lieshout & Roestenberg (2015) highlighted that it is important that microscopic expertise within the field of gastrointestinal parasites is not lost due to this reason. As such, the training of analysts in this skill should be encouraged by public health bodies, despite the movement away

from these specialized and traditional techniques. This will ensure that a level of specialized expertise within public health laboratories is not lost as a highly useful back-up when more advanced techniques fail or are suspected of being incorrect. Within Scotland this is apparent whereby 17 of 19 laboratories still continue to use microscopy during detection of *Giardia duodenalis* (personal communication (HPS, 2016)).

1.8.3 *Treatment of Giardiasis*

Giardiasis is usually self-limiting in most cases, however treatment is recommended, where available following positive diagnosis, due to the possibility of the development

Table 4 - Anti-*Giardia* drugs and their recommended treatment durations, doses and possible side-effects

Drug Name	Adult Dosage	Dose / Day	Treatment Duration	Child Dosage	Dose / Day	Treatment Duration	Adverse effects from drug use
Metronidazole (Flagyl)	250mg	Three times a day	5-7 Days	5mg/kg	Three times a day	5-7 Days	Headache, vertigo, nausea, metallic taste, urticaria, Disulfiram-like reaction with alcohol ingestion. Rarely: Pancreatitis, central nervous system toxicity, peripheral neutropathy, among other effects.
Tinidazole (Fasigyn)	2g	Once a day	Single dosage	50mg/kg (max 2g)	Once a day	Single Dosage	As with Metronidazole.
Ornidazole (Avrazor)	2g	Once a day	Single dosage	40-50mg/kg (max 2g)	Once a day	Single Dosage	As with Metronidazole.
Quinacrine (Atabrine)	100mg	Three times a day	5-7 Days	2mg/kg	Three times a day	7 Days	Nausea and vomiting, dizziness, headache, yellow/orange discolouration of skin and mucous membranes Rarely: toxic psychosis.
Furazolidone (Furoxone)	100mg	Four times a day	5-7 Days	2mg/kg	Four times a day	10 Days	Nausea, vomiting and diarrhoea, brown discolouration of urine, disulfiram-like reaction with alcohol ingestion.
Paromomycin (Humatin)	500mg	Three times a day	5-10 Days	30mg/kg	Total dosage via 3 doses per	5-10 Days	Ototoxicity and nephrotoxicity with systemic administration.
Albendazole (Albenza)	400mg	Once a day	5 Days	15mg/kg/day (max 400mg)	Once a day	5-7 Days	Anorexia, constipation. Rarely: reversible neutropenia and elevated liver function tests. Possibly teratogenic.

Table 4 - This table lists various drugs used when treating *Giardiasis* in patients. Drug and common trade names, concentrations, dosages, treatment duration as well as side effects of the drugs are listed. Nitroimidazoles are the preferred drug for use when treating the disease (Metronidazole, Tinidazole and Ornidazole) and thus are most commonly prescribed by physicians. Metronidazole is dosed at three times a day due to its short retention inside the human body, however Tinidazole and Ornidazole have much longer half-life's and as such require only a single daily dose. Other drugs which can be used tend to have more severe or uncomfortable side effects for the patient and so are not commonly prescribed unless required (table adapted from information on Gardner & Hill, 2001).

of chronic infection (Table 4). Treatments of both acute and chronic giardiasis are carried out using specific medications such as Metronidazole (Flagyl), however a range of choice drugs are available for use. Compounds of nitroimidazole are preferred (Metronidazole, Tinidazole and Ornidazole); however others may be required in different case. Infection within patients can vary extensively, meaning that different drugs may be required to combat individual infections. Similarly due to the all of the drugs having side effects, some may be more suited to individuals than others. Metronidazole is required to be taken most frequently at three times a day, out of the nitroimidazole compounds, due to its high uptake in the body. The other two compounds, Tinidazole and Ornidazole, only need to be taken once a day due to a longer half-life within the body, however have a high degree of success in combating the infection (Gardner & Hill, 2001).

As mentioned, all of the drugs which treat giardiasis in humans are known to have potential side effects; however they are often outweighed by the symptoms of infection and potential risk of developing chronic infection (Escobedo & Cimerman, 2007). Many authors have also reported that when chronic infection of giardiasis occurs it has been seen to be coupled with the onset of Irritable Bowel Syndrome (IMS). Studies have also found that previous infection with *G. duodenalis* increases an individual's chances of developing IBS or chronic fatigue syndrome later in life (D'Anchino *et al*, 2002; Stark *et al*, 2007; Alexander *et al*, 2014; Hanevik *et al*, 2014).

1.9 Project Brief, Outline and Aim

Giardia is a genus of bi-nucleated and flagellated protozoan parasites responsible for causing the globally widespread disease known as giardiasis in humans. The parasite is thought to be the most common cause of protozoan diarrhoea worldwide and as such is an important organism in terms of public and also veterinary health (Sprong *et al*, 2009). Host range includes species close to human lives such as various livestock animals, together with cattle, sheep, dogs, cats and wildlife (Feng & Xiao, 2011). The UK is no exception with regards to this issue and this work will initially review the status of Giaria duodenalis infection within the UK, focussing on similarities between Scotland and England and Wales. Previous public health data shows a clear difference between these two parts of the UK with regards to the apparent occurrence of *G. duodenalis* infection within the two populations. This data will be reviewed along with

relevant publications to allow better understanding of the complex epidemiology of the parasite as well as how the differences between demographics could be caused, looking specifically at between-assemblage infection differences. Selected papers, relevant to this study, will also be reviewed focussing on the presence of *G. duodenalis* in Scottish Waters.

Worldwide, and indeed within the UK, *G. duodenalis* is found in both raw surface waters and even occasionally in finished waters during outbreaks. In the second chapter, the main methodologies in which the parasite is extracted from the environment, water filtration, will be reviewed. This is done in order to identify possible areas of current processes which could be improved by the inclusion of a novel filtration system developed utilizing megasonic waves. Possible benefits of the inclusion of the megasonic system in the existing filtration method will also be justified, before a methodology for megasonic sonication is designed, tested and evaluated for *G. duodenalis*. This section of the work will be based specifically on the methodology of the IDEXX Filta-Max system, a commonly utilized filtration method and one which is recommended by the Drinking Water Inspectorate (DWI) within the UK.

Following on from this section, in the third chapter, work which was carried out investigating the epidemiology of the parasite within Scotland will be reported. Both water samples from locations around Scotland and cattle samples from a farm close to Edinburgh city were investigated for the presence of *G. duodenalis* and where possible, their assemblage was determined. The work will be discussed similarly to the above; with the methodologies described, the results discussed and finally an interpretation of the data conferred.

2. Chapter 2 – Development of Novel Approaches of *Giardia duodenalis* Filtration Elution using Megasonic Sonication.

2.1 Waterborne Disease and Water Regulations

It is no surprise that the quality of drinking water supplied by water industry sites in the developed world is often monitored to prevent water contaminated with pathogens being consumed by the public. Currently there are regulatory requirements for water within the UK for a similar protozoan species, *Cryptosporidium*, which has been extensively monitored in water since their introduction of the rules under the Water Supply (Water Quality) Regulations 1999, SI No. 1524. These regulations state that treatment works must have risk assessments carried out to accurately quantify the risk of *Cryptosporidium* oocysts from their waters. If a significant risk of infection is present the supplier must ensure appropriate steps are in place to remove the contaminant from the water until safe to drink. Although expensive to both maintain and establish, these regulations have had a positive impact on reducing outbreaks of *Cryptosporidium* in the public and data collaborated by Robertson & Lim (2011c), on work by Lake *et al* (2007), suggests that in North England 905 cases (~7000 infections) were prevented since its implementation to 2007.

Although not monitored within the UK, *G. duodenalis*, amongst a large number of other water contaminants, is extensively monitored within the USA. Following the EPA Safe Drinking Water Act (SDWA) in 1997 *Cryptosporidium* and *Giardia* became extensively monitored and regulated using US EPA Method 1623 (Method 1623.1, 2012). These introductions have been very effective in reducing outbreaks within the USA where there have been more reported outbreaks of giardiasis than anywhere else worldwide (Robertson & Lim, 2011d). Monitoring of *G. duodenalis* in the UK in the same manner which has already been established for *Cryptosporidium* would certainly be costly and time-consuming; however it would both benefit public safety upon its implementation, as well as allow increased understanding of *G. duodenalis* epidemiology within the UK through catchment data collection. Reasoning for a lack of established regulations is that it is thought that *G. duodenalis* does not occur, at least from past historical data, or to cause very few outbreaks in comparison to other waterborne contminants within the UK and therefore causes little perceived threat. Data

sets from Public Health officials do however report cases of *G. duodenalis* infection within the UK and by Public Health England and Wales in 2013 it was estimated that there were 3624 reported cases of giardiasis (https://www.gov.uk/-guidance/giardia). In the same year Health Protection Scotland reported only 167 cases of *G. duodenalis* infection (http://www.hps.scot.nhs.uk/documents/ewr/pdf2015/1537.pdf). These figures highlight that the situation in Scotland is different than in England and Wales, although reasons for this are largely unknown and that large scale epidemiology work needs to be carried out within the UK.

It is thought that dynamics of *Giardia* infection are partially to blame for the lack of epidemiological understanding. Delay between point of infection and clinical disease, varying clinical symptoms displayed in individual infections result in potential large-scale under-reporting of the parasite throughout the UK, and indeed the world. The symptoms which giardiasis manifests are very similar to a large number of other pathogens, notably in the acute phase of disease (diarrhoea, nausea, cramps etc.) (Jakubowski, W., 1988). This often leads to general practitioners failing to identify *G. duodenalis* as a potential cause of illness, or miss-diagnosis. If the practicioners do not suspect the parasite as the cause of illness and test for it, cases cannot be diagnosed. Cases are also known to be asymptomatic often, whilst shedding of cysts will still occur (Gardner & Hill, 2001). Due to this an accurate level of *G. duodenalis* infection within the UK, is still largely under debate.

2.1.1 *Current Filtration of Giardia duodenalis from Water Sources*

Filtration of *G. duodenalis* from water samples is currently carried out using the same methodology as that for *Cryptosporidium* in the UK - the FiltaMax system. Only different Immunomagnetic separation (IMS) reagents are required. (UK Environment Agency (2010), page 82). The nature of recovering oocysts/cysts from Filta-Max filters is such that it must be carried out by specialized analysts with a high degree of training to ensure maximum sample recovery which is crucial when attempting to understand the threat of a water supply to public health. Many parts of the process currently must be carried out manually by the analyst, which can be affected by human error, leading to further potential losses or inconsistencies. A Filta-Max automatic wash station is available and is commonly utilized by water companies in an attempt to both reduce the level of manual activity required by analysts processing filter samples, as well as standardizing the elution process by removing human involvement. Unfortunately, this

wash station is only designed to assist in the initial elution processes and cannot be used for the latter stages of the method, meaning variation due to human error still exists as well as the time involved in the manual activity. Current filtration recoveries for *G. duodenalis* cysts in reagent grade water is said to be between 41-70%, referring to the UK Environment Agency (2010), something which has been confirmed by other authors, with mean recoveries of 49.8 +/-5.4%, 56.7+/-22.2%, (Wohlsen *et al* ,2004; DWI Report 70-2-155, 2003).

It has also been speculated that *G. duodenalis* cysts, being not as resistant as *Cryptospridium*, may be damaged or destroyed by the process of filtration and/or elution using the Filta-Max System (currently recommended for filtration of *Cryptosporidium* and *Giardia* within the UK and used internationally (DWI Report 70-2-155, 2003). This was noted as a possible source of failure to create a dual-monitoring method for both *Cryptosporidium* and *Giardia* in 2002, which was investigated by Severn Trent Labs on behalf of the Drinking Water Inspectorate (DWI) in the UK (DWI Report 70-2-155, 2003). Robertson & Lim (2011d) did however suggest that this was an odd result as many published work previously has managed to recover cysts without this problem and suggested other causes. Interactions between the environmental water samples and IMS reagents were suggested to be a possibility for low recoveries. Since the early 2000's there has been no shortage of methodologies for filtration of parasites from water sources for research purposes (Wohlsen *et al*, 2004; McCuin & Clancy, 2003). This means that practicality, speed and implementability are now equally as important as simply recovery alone to adequately meet filtration needs (Ferguson *et al*, 2004). Similarly cost effective, reliable and repeatable methodologies of both initial filtrations from water sources as well as final elution of parasites from filtration matrixes are paramount when working to protect public health and generate reliable epidemiological data. For this reason it is of key importance when attempting to improve current standards of water testing for *G. duodenalis* that these factors be implemented in the filtration process where possible. The above mentioned DWI report highlights the need for further developments during the filtration process which can reliably recover *G. duodenalis* cysts from samples and also be combined with the detection of other species effectively. A novel method involving utilizing megasonic sonication to elute parasites from filters has the potential to fill this niche.

2.2 Megasonic Sonication and Incorporation into Giardia duodenalis Filtration

Megasonic sonication operates in a similar way to that of ultrasonic sonication, however a much higher frequency of sound waves are used. Typically megasonic sonication involves a frequency of over 1 Mhz created by a piezoelectric transducer which is placed within a fluid filled container. When powered, the transducer creates high frequency sound waves which move through the fluid and oscillate through maximum and minimum pressures at points along the wave. At the point where the minimum pressure along the wave is below the vapour pressure of the liquid bubbles form; these bubbles then collapse upon exposure to the maximum pressure of the wave. This formation and implosion of bubbles creates a much kinder elution process compared to that of ultrasonic sonication, due to the bubbles being smaller in size and thus creating less local turbulence when collapsing (Kerrouche *et al*, 2015; Chitra *et al*, 2004). In an attempt to improve the current methods of *G. duodenalis* filtration using the FiltaMax system, we have incorporated megasonic sonication into suitable steps of The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14 standard protocol of *G. duodenalis*, which has potential to improve the standard method.

Megasonic sonication has shown promising results in elution of *Cryptosporidium parvum* oocysts from Filta-Max matrixes, as reported by Kerrouche *et al* (2015). The method allowed recoveries of the common waterborne pathogen comparable to that of the Filta-Max methodology (commonly used within water testing internationally, including the UK (The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14). The megasonic technique however displayed a range of benefits over the current method. In this chapter I investigate if the same benefits seen when eluting *Cryptosporidium* by the authors in *Kerrouche et al (*2015) are evident when used to elute *G. duodenalis* from the FiltaMax system.

The standard method of elution is centred on removal of parasite embedded within a filter using an elution buffer (see Appendix D: 5.4.2). This buffer which then contains the parasite is reduced in volume by various concentration steps, aiming to keep the parasite sample within it throughout the various volume reducions. Initially 600ml elution buffer is added into the FiltaMax wash station and the filter module is allowed to expand and washed in the buffer. The resulting eluent (now containing parasite within the sample) is then concentrated through a filter membrane in a concentrator tube via a hand pump, leaving around 30ml above the membrane to avoid

drying it out. This step is repeated for a total of two washes, with the resulting eluent being around 30ml which is stored for later use (the first eluent volume from the first wash is added into the concentration of the second wash). The membrane is then washed to remove any cysts which may have become embedded within it during the concentration steps (the majority of cysts should however have been within the eluent remaining above the membrane). The membrane is washed twice and then added into the same storage tube as the initial concentrations. Areas of megasonic incorporation were as follow: washing of the filter module to remove parasite and stages thereafter (section 2.3.4), concentration of the eluent which was used to remove parasite from the filter module (section 2.3.3) and finally the removal of parasite from the membrane (section 2.3.2). A complete method was then developed for the incorporation of the megasonic technique into the standard method (section 2.3.5).

Here I attempt to reduce the level of manual labour required per filter, as previously observed possible for *C. parvum,* as well as maximising the time efficiency of processing a filter containing *G. duodenalis* cysts whilst still achieving satisfactory recovery levels. I also indirectly investigate if the megasonic sonication method is more suitable than the standard method with regards to damage and effects on *G. duodenalis* cyst stability during elution. A previously mentioned DWI study reported cyst destruction during the processing as a possible explanation for poor/variable recoveries observed with *G. duodenalis* from filter matrices currently in use within the UK (DWI Report 70-2-155, 2003).

Megasonic sonication has been highlighted as a potential basis for a fully automated system of parasite recovery from filtration matrixes by Kerrouche *et al* (2015). This combined with a decrease in volume of reagents required and a notable reduction in manual time requirements promotes the potential that megasonic sonication has for future implementation in the water industry.

2.3 Materials and Methodologies

Three steps of the original method described in The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14 were investigated for the inclusion of megasonic sonication for improvement via megasonic sonication. A complete method for using megasonic sonication was developed using ideas both from Kerrouche *et al* (2015) and based on evaluating the experience from shortfalls from these initial three areas of potential for inclusion of megasonic sonication, as mentioned in the previous section.

In all experiments samples were spiked appropriately with EasySeed suspensions, which contained 100 gamma-irradiated *G. duodenalis* cysts each (TCS Biosciences, UK), except with regards to the viability/integrity assay of cysts in which live cysts were purchased for use (Waterborne Inc, USA). Unfortunately EasySeed suspensions were only purchasable in the form of inactive cysts (via gamma-irradiation) and so it was decided that these would be used for the work due to their high reliability of cysts/suspensions (99 +/- 1 cyst). The megasonic transducer used for this work was supplied by Dr Helen Bridle from Heriot-Watt University, purchased from Sonosys (Sonosys) and FiltaMax modules and membranes were purchased from IDEXX (IDEXX Company). Each sonication step was carried out for 20 minutes as standard, an exposure time taken from Kerrouche *et al (2015).* The resulting slides from each experiment were then stained using a monoclonal antibody as per manufacturer's instructions (*Giardia*-Cel Reagent – TSC Biosciences) and a nucleic stain (DAPI (4',6-diamidino-2-phenylindole) – Fisher) at a concentration of 2mg/ml diluted to 1:5000 in Phosphate Buffered Saline (PBS – Moredun Research Institute (MRI) Stores Dept.). Cysts were then counted using fluorescence and DIC (differential interference contrast) microscopy and the total numbers of cysts recorded.

2.3.1 *Viability Assessment of Megasonic Exposed Cysts*

Cysts were also exposed to megasonic waves prior to their viability being assessed using a propidium iodide (PI) assay test. This was used to assess if megasonic waves had an effect on the viability and integrity of the *G. duodenalis* cysts both on initial sonication and over a prolonged time period. Propidium Iodide is a nucleic acid dye which can cross over the biological membranes of only dead organisms. Viable cysts, having a functional biological membrane, do not allow the dye to cross into the

organism internal contents and therefore are not stained by it. In dead cysts the dye stains the internal contents of the cyst (Jones & Senft, 1985).

Table 5. Propidium Iodide Viability Assay for Megasonic Exposed *G. duodenalis* Cysts

Sample/Replicate Number	Time of Exposure (Megasonic)	Day of Viability Assay (from day of sonication)
1	20 minutes	Day 1
2	0 Minutes	Day 1
3	20 minutes	Day 7
4	0 minutes	Day 7

Table 5. This table details the experimental setup for investigations into how the megasonic waves affect *G. duodenalis* cyst viability. The affect on cyst viability was measured using the vital dye Propidium Iodide. The dye binds to the nucleic contents of cysts which are non-viable which then fluoresce with a red glow using a fluorescence microscope with a filter set to a specific wavelength (535-nm excitation, >590-nm emission). As the dye cannot cross functioning biological membranes viable cysts are not stained and this therefore highlights the levels of viable/non-viable cysts within the sample. Cysts were also stained with FITC (Fluorescein isothiocyanate) cMAb to allow easier identification of cysts on the slide. The viability assay was carried out on the same day (day 1) as exposure to megasonic waves occurred in samples/replicates 1 and 2, where as samples/replicates 3 and 4 had their viability assay carried out 7 days from exposure (day 7).

Viewing the cysts using a UV microscope with a filter set to a specific wavelength (535-nm excitation, >590-nm emission) allowed differentiation between stained non-viable cysts and not stained viable cysts. DAPI (4', 6-diamidino-2-phenylindole) was also included in the sample staining as it intercalates with trophozoite DNA within the cyst, allowing further confirmation as to the presence of trophozoites being present within the cyst shell. DAPI stain can be seen to fluoresce sky-blue within the cyst where DNA is present in the nucleus (within the trophozoites) when exposed to an ultraviolet wavelength of 350-nm excitation, >450-nm emission via filter block using a UV microscope. Finally the cysts were stained with a monoclonal anti-body specific for *Giardia* species (*Giardia*-cel (TCS Biosciences) which made locating cysts on the slide for PI and DAPI stain assessment easier. A total of $2.5x10^5$ *G. duodenalis* cysts in 1ml were added into 19ml of ultrapure water before 5ml of the suspension being added into a single Filta-Max plastic bag. A total of four replicates were prepared, of these, two bags containing *G. duodenalis* cysts (bags 1 & 3) were exposed to megasonic waves for 20 minutes and the remaining two (bags 2 & 4) were not exposed as controls (Table 5).

Megasonic exposed sample/replicate 1 and non-megasonic exposed sample/replicate 2 had their viability compared immediately following the megasonic exposure of sample/replicate 1. This was done to investigate if the megasonic waves had any immediate effect on cyst viability. To do this, 1.5ml of sample/replicate 1 & 2 were each transferred into separate 2ml cryotubes and concentrated using a centrifuge at 500g for 7 minutes. The supernatant in each was then removed down to a volume of ~100µl and 900µl of HBSS (Hanks Buffered Salt Solution, MRI Stores dept.) was added before further centrifugation at the same speed/duration as above.

The supernatant was then removed down to ~100 µl again and to each sample 10µl of Propidium Iodide was added (1mg/ml in 1xPBS) before being incubated for 1.5hrs at 37^{o}C. Following this 100µl of *Giardia*-cel CmAb (TCS Biosciences) was added neat, as per manufacturer's instructions, and incubated for a further 15 minutes at 37^{o}C. Each sample was then centrifuged again at the same speed/duration as previous and the supernatant in each again reduced to 100µl, followed by a 900µl HBSS wash to remove excess Propidium Iodide and FITC CmAb. 10µl was then pipetted onto a microscope slide, enclosed with a cover slip and sealed with nail varnish. The slide was then read using an Olympus BX50F & BX-FLA attachment equipped with Nomarski Differential Interference Optics. Each count consisted of a total of 100 cysts and the number of non-viable (PI+ve) and viable (PI-ve) cysts were counted and recorded. Cyst intergrity was also observed.

2.3.2 Direct Membrane Seeding

This section of the work allowed an understanding of whether the megasonic exposure would satisfactorily recover cysts potentially embedded within the FiltaMax membrane which is used to filter and concentrate the eluent used to wash the FiltaMax module. The base of the FiltaMax concentrator tube (without the tube) was fitted with a FiltaMax membrane and a hand pump before being primed using PBST (Phosphate Buffered Saline / Tween 20). The 100 cyst spike (gamma-irradiated cysts) was then made up to 5ml using PBST, passed through the membrane using an appropriate pipette whilst applying pressure using the hand pump.

A wash of 2ml PBST was then added to the tube and thoroughly mixed before being passed through the same membrane. Care was taken when passing the spike through the filter to ensure that pressure did not exceed levels comparable to the standard method.

Figure 4 – Diagram of the Megasonic Bath Setup

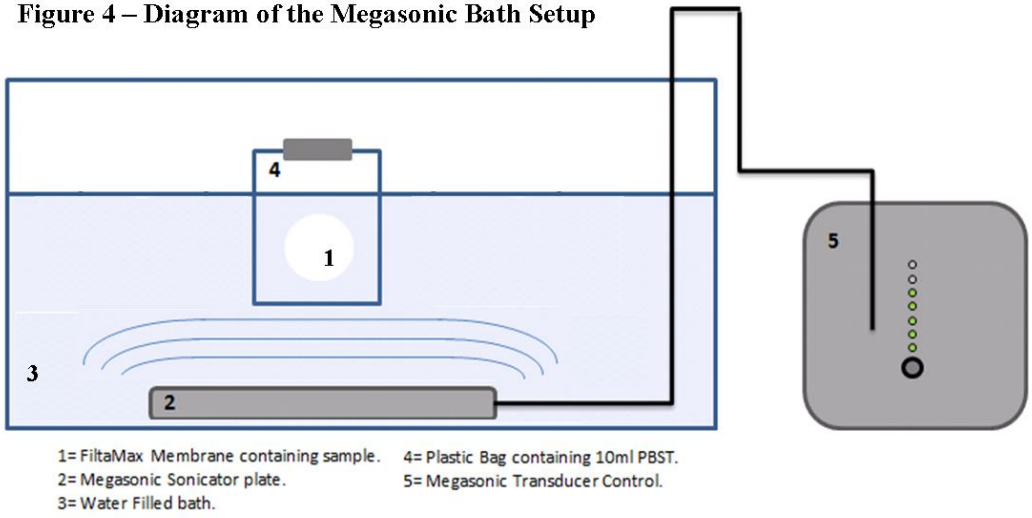


Figure 4. A diagram showing the setup of the direct seeding experiment megasonic procedure. The samples can be seen submerged in the water bath being exposed to the megasonic waves produced by the megasonic sonicator plate. Samples were sonicated for 20 minutes.

Six sample membranes were prepared in this manner and from these three were processed using the standard rubbing method using 2x5ml PBST washes in a plastic bag (as supplied with IDEXX FiltaMax membranes) as controls with the eluent from each wash transferred into a single labelled centrifuge tube. Following this, the total 10ml from the control filter membranes were each made up to 50ml using ultrapure water and centrifuged as standard, prior to following the protocol onto the IMS stage of processing. The final three sample membranes were processed using megasonic sonication inside of a water bath, instead of the standard method of rubbing. These sample membranes were placed in a standard plastic bag, as above, containing 10ml PBST and taped to the side of the megasonic bath, ensuring the total eluent wash was immersed within the water (Figure 4).

Samples were sonicated for 20 minutes, which was based on the paper by Kerrouche *et al* (2015) as an optimum exposure time. The 10ml sample was then transferred directly from the plastic bag into a Leighton tube and from this point IMS was carried out following the standard protocol, using an Invitrogen Dynabeads® G-C Combo kit. An additional sample was also processed as a control (non-megasonic method) for centrifugation, to assess if this had any effect on the cysts recovered via this method. In this work a single sample was processed following the standard protocol,

however the 2x5ml washes were placed directly into a Leighton IMS tube and processed using IMS.

2.3.3 *Seeding into Increased Eluting Volume (1200ml PBST)*

The direct seeding experiments investigated the abilities of megasonic sonication to remove *G. duodenalis* cysts from membranes upon direct seeding. This section of work allowed insight into how the volume of eluent containing the cysts would affect the abilities of the megasonic exposure to recover cysts from the membranes. Six samples were prepared in the following manner. A FiltaMax concentrator tube base was fitted with a FiltaMax membrane and the concentrator tube was screwed in as would be done as standard. To this tube 600ml PBST was added in accordance with the standard protocol and a spike of 100 *G. duodenalis* cysts was added (as above made up to 5ml and followed by a 2ml wash). The sample was concentrated through the membrane as standard using a magnetic stirrer. Following a small amount of liquid remaining above the membrane, an additional 600ml PBST was added to the tube (to represent the second wash as per the standard protocol) and concentrated down to the same low level (now totalling 1200ml PBST used). The magnetic stirring bar was removed and washed using a small amount of PBST to remove any cysts that may have been attached. The sample was again concentrated briefly until a low level of eluent remained above the membrane surface. From this point three samples were processed as per the standard methodology and three were processed using megasonic sonication.

Following the standard method, the sample was decanted from the concentrator tube into a centrifuge tube and the membrane was added into a bag with 2x5ml PBST washes, before the washes being added into the same centrifuge tube as the concentrator tube sample eluent. These were then made up to 50ml and centrifuged and prepared for IMS processing following the standard method.

The megasonic method involved direct transfer of the remaining concentrator tube eluent into the plastic bag which would contain the membrane for processing. The concentrator tube was then washed briefly with 10ml PBST and again decanted into the plastic bag. The membrane was then removed from the concentrator tube and carefully placed into the same plastic bag (now containing ~50ml eluent). This was then sonicated in the same method as the previous experiment, but with 50ml PBST as opposed to 10ml as previous (Figure 4). Following the megasonic exposure the eluent

from the bag was transferred into a centrifuge tube and centrifuged and prepared for IMS processing as standard.

2.3.4 *Seeding into Sponge Filter Matrices*

Filters were prepared for seeding as standard and 1000L of water was passed through them. Half way through the filtration (500L) a spike of 100 *G. duodenalis* was injected into the filter. The 10ml spike was created by drawing the cyst suspension (made up to 5ml), plus a 2ml wash, into a 10ml syringe and adding ultrapure water to make the spike up to 10ml total volume. Syringes were numbered and filter housings unique identifiers were documented to allow traceability following spiking.

Following this, filters were either processed using a megasonic method or the standard method, as can be found in The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14. The megasonic method involved removing the filter module from the filter housing and removing the screw holding the filter membrane together. The filter sponges, now loose, should be placed into a large plastic bag containing 1200 PBST. The sponges should be separated from each other when inside the bag when possible to promote removal of cysts from the matrices. The plastic bag, now containing the separated sponge filters, was placed into the megasonic bath and sonicated for 20 minutes set to 2MHz. Following this, the bag was removed from the megasonic bath and the liquid poured into a concentrator tube containing a magnetic stirrer. This was then concentrated down until a small amount of liquid remained above the membrane. The bag was then cut at one corner and squeezed to remove any remaining PBST containing cysts into the concentrator tube. The liquid was again concentrated down to a small level above the membrane. The liquid was then poured into a centrifuge tube for later use, along with a small amount of PBST to wash the concentrator tube. From this point the membrane was processed as usual following the The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14.

2.3.5 *A Complete Procedure of G. duodenalis Elution using Megasonic Sonication*

The filters containing known quantities of *G. duodenalis* cysts were prepared in the same manner as described in the section above, using suspensions containing 100 gamma-irradiated cysts. Six filters were prepared in total, with three acting as controls to be processed according to the standard methodology found in The UK Environment

Agency (2010) Microbiology of Drinking Water: Part 14. The final three were processed using a methodology developed to incorporate megasonic sonication fully into the elution process. Adaptations were made to the procedure following results obtained from previous work, which included additional wash steps being incorporated into steps in the method. When sonicating filter sponges (again in the same method described in the previous section) a PBST volume of 1000ml was utilized, instead of 1200ml. Following sonication, the PBST was poured from the bag into the concentrator tube (containing a filter membrane) and the sponge disks were wrung out and then concentrated down to a lower volume using the hand pump. A final wash of 200ml PBST was then added to the bag to recover any cysts still remaining in the plastic bag or sponge disks. The 200ml PBST wash was then manoeuvred briefly inside the bag, before being poured into the same concentrator tube as the 1000ml and the disks wrung out again.

The concentrator tube containing the membrane and eluent was then drained down slowly to a point in which the membrane was dry, being careful not to damage the membrane itself. The membrane was then removed and added into a plastic bag containing 8ml PBST, which was then placed into the megasonic bath to be sonicated for 20 minutes. This 8ml eluent was then transferred directly into an IMS Leighton tube and a further 2ml wash of PBST was added to the bag to recover any cysts which had been left behind in the first wash. This 2ml wash was then removed from the bag and transferred into the same IMS Leighton tube, totalling 10ml, to be processed as standard following The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14.

2.4 Results and Discussion

2.4.1 *Viability Assessment of Megasonic Exposed Cysts*

Cysts were exposed to megasonic waves in order to determine if their viability would be compromised by the exposure to megasonic waves. It was found that the megasonic waves did appear to have an impact on the viability of live *G. duodenalis* cysts; however their structural integrity did not appear affected by this (from visual observations). Both of the samples exposed and not exposed to megasonic waves on day 1 had a mean of 81.5 and 84 viable cysts, respectively, which was not found to be

significantly different (p=0.722). This was not seen on day 7 however, as the exposed and non-exposed cysts had mean viability of 64 and 74.5 respectively; this difference was statistically significant (p=0.041) (Table 6). This highlights that over the 7 days from exposure the cysts had become less viable compared to the control group.

Although there were noted to be significant reductions in the viability of the samples after 7 days of exposure to the megasonic waves compared to the controls, day of exposure samples were not significantly different. The small loss of viability seen one week from megasonic exposure, although significant, is not seen as a large issue as real world water samples should be prepared by the water laboratory as soon as possible. An increased time between periods of sampling and processing leads to

Table 6 - Viability Assessment using Propidium Iodide and Statistical Significance of Results

Method	Viable Cysts			Dead Cysts			Standard Deviation	Statistical Significance
	Count 1	Count 2	Mean	Count 1	Count 2	Mean		
Megasonic Day 1	78	85	81.5	22	15	18.5	4.95	P=0.722
No Megasonic Day 1	79	89	84	21	11	16	7.07	
Megasonic Day 7	63	65	64	37	35	36	1.41	P=0.041
No Megasonic Day 7	74	75	74.5	26	25	25.5	0.71	

Table 6 - This table shows the results of viability assessment of *G. duodenalis* live cysts when stained with propidium iodide (PI). In each count performed a total of 100 cysts were included and separated into 'live' or 'dead' based on their uptake of the stain. Dead cysts became stained by PI, where as live cysts did not. Standard deviations apply to both live and dead cyst counts as they each form a proportion of the total count (100). It was found that there was no statistically significant difference between megasonic exposed cysts and control cysts on day 1 (p=0.722), however there was a difference between megasonic exposed cysts and control cysts on day 7 (p=0.041). Statistical tests were carried out using a One-Way ANOVA.

decreased recovery and so water authorities will try their upmost to process samples as soon as possible. Furthermore the fact that the cysts appeared to be less damaged/broken during elution from filter materials outweighs this higher percentage of non-viable cysts from megasonic exposure as they can still be used for DNA based work as well as identified microscopically.

2.4.2 *Megasonic Elution from Directly Seeding Membranes*

The megasonic elution of cysts from spiked filter membranes showed promising results, notably with regards to the difference between mean standard and megasonic cyst shell

levels which were 33 and 1, respectively (Table 7 and Figure 5). This part of the study assessed the capabilities of the megasonic sonication to remove cysts which may be embedded in a filter membrane. This is important as it will occur during routine filter processing using the standard method and so investigations into how the megasonic method performs at this step is vital, compared to the standard method.

Recoveries from both methods are comparable when including cyst shells within the total. However, if these are not to be included, the megasonic procedure is

Table 7 – Results of Standard and Megasonic Techniques used on Directly Seeded Membranes.

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	64	25	39	25.0%	64.0%	60.9%
	2	53	19	34	19.0%	53.0%	64.2%
	3	66	41	25	41.0%	66.0%	37.9%
	Mean	61	28	33	28.3%	61.0%	54.3%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	61	59	2	59.0%	61.0%	3.3%
	2	67	67	0	67.0%	67.0%	0.0%
	3	64	64	0	64.0%	64.0%	0.0%
	Mean	64	63	1	63.3%	64.0%	1.1%

Table 7 – Recoveries of FiltaMax membranes seeded with *G. duodenalis* cysts using megasonic and standard methodologies. Each experiment was spiked with 100 cysts total. It can be seen that the megasonic methodology has an advantage with regards to recovering intact cysts, despite the total numbers of cysts + shells in both cases being largely comparable. The numbers of intact cysts averaged at 28% in the standard method replicates, compared to around 63% intact cysts using the megasonic method. A difference in the number of shell ratios present when comparing both methods was also found, with the standard method having a mean of 54% shells compared to the megasonic method which had 1%. Shells were largely absent in the megasonic methodology. Where appropriate totals have been rounded to the closest cyst.

significantly (p-value of 0.007 using a One-Way ANOVA) better at recovering intact cysts (standard method recovered 28% vs megasonic which recovered 63%). The standard procedure within a water laboratory is that a cyst shell is regarded as a non-reportable object and thus not counted as an organism. These are often reported as '*Giardia* like-bodies' or 'glbs' in a comment or side note for the sample, but not included in a total count of cysts within the sample as they do not pose a risk to public health. Due to this, the integrity of cysts recovered from a water samples is of crucial importance in assessing threat, to public health within a sample from a catchment. This work highlights the base efficiency of megasonic sonication when recovering cysts from

a membrane and demonstrates the benefits which could be incorporated into the current standard procedure for *G. duodenalis* filtration.

A control was also included to ensure that the centrifugation step was not responsible for producing the damaged cysts in the standard process. It was found that cysts were similarly as damaged following the standard process when the centrifugation step was skipped (41% of total recovered objects being shells), suggesting that the

Figure 5 - Direct Seeding onto Membrane Experiment

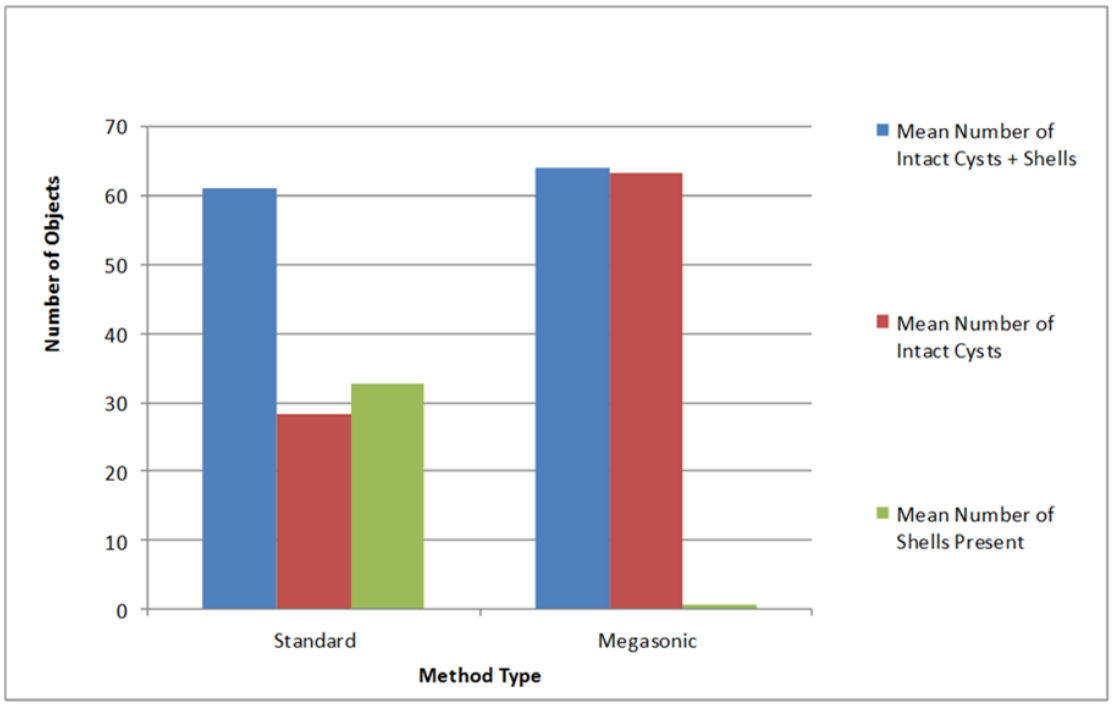


Figure 5 – A graph of cyst recoveries from the direct seeding experiments. It can be seen that a comparable level of mean total objects were recovered by both the standard and the megasonic methodologies (mean 64 cysts by Megasonic vs mean 61 cysts by Standard). However the mean numbers of intact cysts in the standard method was reduced (mean 28 intact cysts) which had an increased number of shells (mean 33 by standard method vs mean 1 by megasonic method) when compared to the megasonic method (mean 63 intact cysts).

elution process itself was the cause of the damages. This work would suggest that the megasonic wave action of eluting *G. duodenalis* cysts from membranes is much gentler compare to the standard rubbing method used to remove potentially embedded cysts from the membranes.

2.4.3 *Seeding into Increased Eluting Volume (1200ml PBST)*

This part of the work aimed to investigate how a higher volume of eluent containing cysts being passed through the membrane would affect recovery of cysts using

megasonic sonication. This part of the work was a direct repeat of the previous section of the study, with the only difference being an increased eluting volume. This was done in an attempt to reflect the higher volume of eluent required in the current protocol employed by water companies, making the process more comparable. The recoveries of the megasonic work here were not as promising as the initial seeding experiment (Table 8 and Figure 6). In this method of operation the standard procedure offered higher recovery rates of both intact cysts (69% versus 55%) and total count of cysts + shells (78% versus 55%), additionally the megasonic approach appeared more variable.

Table 8 – Results of Standard and Megasonic Techniques Used on Seeded Membranes with an Increased Eluent Volume

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	78	72	6	72%	78.0%	7.7%
	2	84	71	13	71%	84.0%	15.5%
	3	73	65	8	65%	73.0%	11.0%
	Mean	78	69	9	69%	78.3%	11.4%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	47	47	0	47%	47.0%	0.0%
	2	48	48	0	48%	48.0%	0.0%
	3	69	69	0	69%	69.0%	0.0%
	Mean	55	55	0	55%	54.7%	0.0%

Table 8 – The results of the experiments performed using an increased volume of PBST (1200ml). It can be seen that although the number of cysts recovered by the megasonic technique is not as high as the standard method (mean total number of cysts + shells by megasonic method is 55, compared to 78 by standard method), there is a clear difference between the shell levels recovered from both methods. The numbers of intact cysts recovered in the standard method is greater than the megasonic, however this method had 0 shells recovered, compared with the standard method which had a mean of 9 shells recovered. Where appropriate totals have been rounded down to the closest cyst.

However, greater cyst damage was observed with the standard approach (9% of the total count being shells for the standard method compared with 0% when using megasonic elution). Although not as clear cut as in the previous section when using a using the direct membrane approach, this shows that the megasonic method is still better at avoiding cyst destruction.

Losses throughout the process are thought to be the main reason for lower recoveries observed between megasonic and the standard method. The direct seeding has fewer areas for potential for losses compared to this experiment which could explain the difference observed between the two tests. For example, an increased surface area of eluent in contact with the plastic concentrator tube, along with increased dispersion of eluent on the membrane when concentrating due to the larger volume, could have made an impact on recoveries.

Figure 6 - Seeding into Increased Eluting Volume (1200ml PBST)

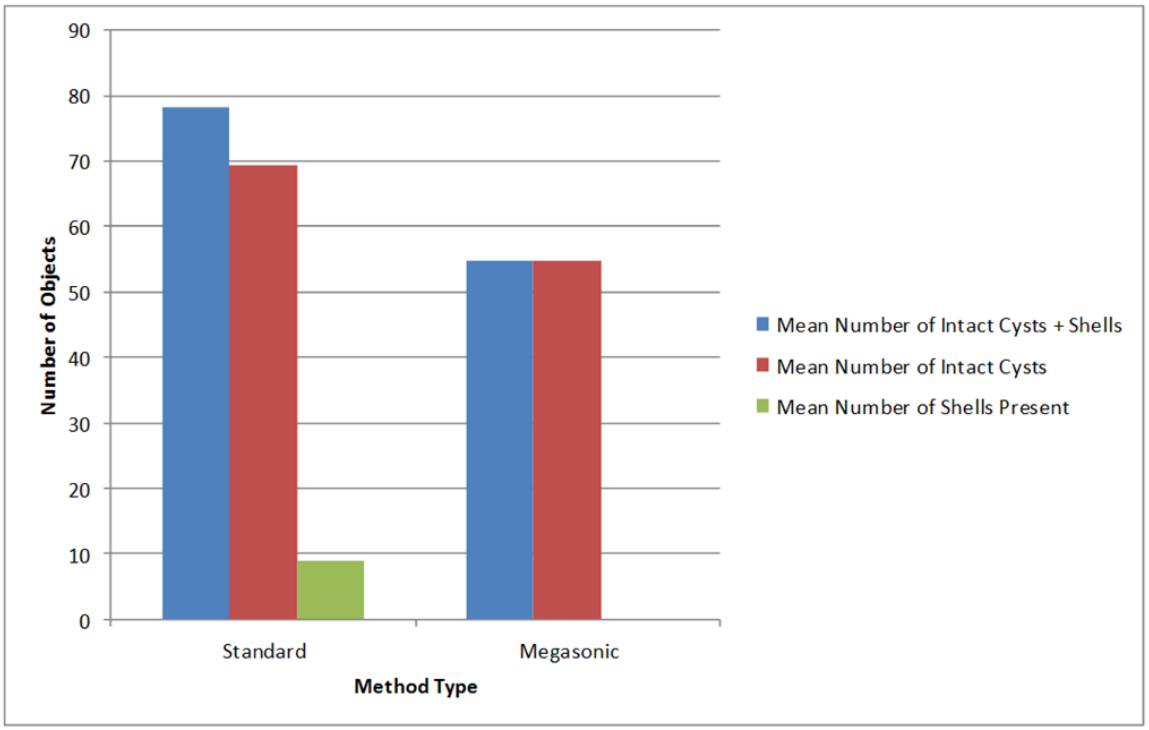


Figure 6 – A graph of recoveries from standard and megasonic experiments using an increased volume of eluent. From the graph it can be seen that the megasonic method was not able to recover a similar amount of cysts when compared to the standard method in this experiment (mean 69 intact cysts by standard method vs mean 55 cysts by megasonic method). There were however an increased number of damaged cysts in the standard method, compared to the megasonic method in which there were none (standard method had a mean of 9 shells while megasonic had 0).

In the increased eluting volume the cysts also appeared to be not as damaged as in the direct seeding experiment, however there were still more shells observed in these

controls than the corresponding samples exposed to megasonic sonication. The standard methodology utilised by The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14 has been optimised to achieve the most efficient recoveries, and as the new method of megasonic sonication has seen limited use with regards to elution of pathogens it is obvious that optimisation is required for increased eluent volumes. Incorporation of additional wash steps into this procedure could potentially increase the lower than expected recoveries achieved by megasonic assisted elution in these experiments.

2.4.4 *Megasonic Elution from Sponge Filter Matrices*

The investigations into the efficiency of removing *G. duodenalis* cysts from filter sponges using megasonic sonication yielded acceptable results, considering the currently limited optimisation (Table 9 and Figure 7). This step investigated whether megasonic sonication could effectively remove *G. duodenalis* cysts, compared to the standard method, which are trapped within the filter matrix. This section is potentially

Table 9 - Results of Standard and Megasonic Techniques used on Seeded FiltaMax Sponges

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	25	21	4	21.0%	25.0%	16.0%
	2	21	17	4	17.0%	21.0%	19.0%
	3	23	21	2	21.0%	23.0%	8.7%
	Mean	23	20	3	19.7%	23.0%	14.6%
Megasonic	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
	1	15	15	0	15.0%	15.0%	0.0%
	2	17	17	0	17.0%	17.0%	0.0%
	Mean	16	16	0	16.0%	16.0%	0.0%

Table 9 - The results from these experiments show that the megasonic method is not currently as effective using the current protocol compared to the standard technique. Higher recoveries were achieved using the standard technique compared to the megasonic method. Only two replicates were reported for the megasonic method as one was lost during processing. It can be seen however that the megasonic did perform well compared to the standard process if only intact cysts are reported. Here we see that the megasonic process yielded a mean of 16% compared to the mean of 20% from the standard process. As the megasonic process has yet to be fully optimised and is in its infancy, this shows promise for its use. Where appropriate totals have been rounded down to the closest cyst.

the most important of the entire method, because if cysts are not removed during this step, then they cannot be recovered in later steps.

Unfortunately a replicate of the megasonic samples was lost during processing, thus was not able to be included in the results, due to a limited number of enumerated suspensions this was unable to be repeated. It was thought however that as the two samples which were processed fully were roughly similar in value, that this gave an indication of the abilities of the megasonic waves when eluting parasite at this stage. Additional testing would have however been desirable within this section of the work. Filter recoveries were close in value when cyst shells are omitted from inclusion (should not be reported as cyst by water authorities, would be reported as a *Giardia-like* body) in the totals and with further optimization of the megasonic method increased recoveries may be possible. In this experiment the mean intact cyst recovery was 19.7% for the standard method, compared to a lower 16% for the megasonic. Comparing the number of intact cysts in both methods there was found to be no significant difference using a one-way ANOVA (p=0.134). Similarly to the previous experiments, the megasonic method had lower shell levels recovered compared to the standard method (mean of 0 vs

Figure 7 - Seeding into Sponge Filter Matrices

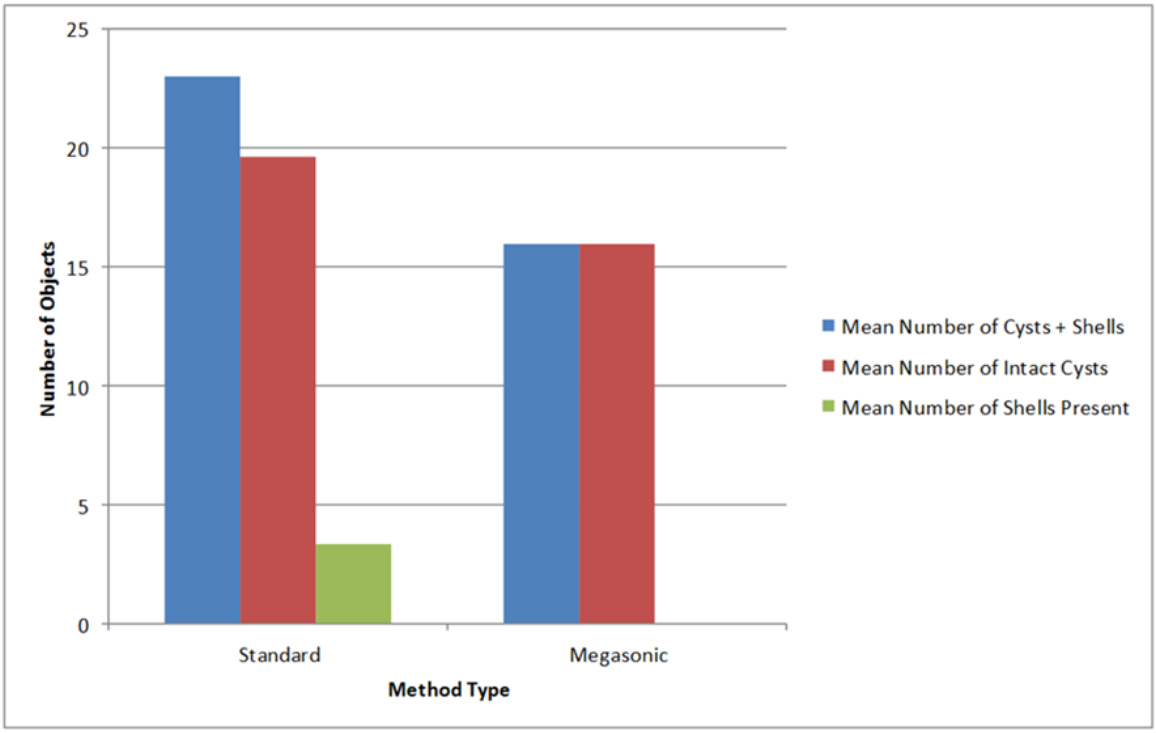


Figure 7 – A graph of recoveries from standard and megasonic methods when seeding directly into sponge filters. It can be seen that the standard method had an increased recovery for both mean number of cysts + shells (23 objects total) and also for mean intact cysts (20 cysts) compared to the megasonic method (both 16 cysts respectively). It can also be seen that the number of shells present in the standard method sample mean is slightly higher than the megasonic method (mean of 3 shells vs 0 shells, respectively).

mean of 3). As in the previous section a main area of losses within the technique is suspected to be caused by the lack of wash steps involved. Inclusion of wash steps

within identified areas, such as when removing the eluent from the megasonic bag may help to increase the recoveries of *G. duodenalis* cysts. It can be seen from Figure 7 that the recovery of cysts from the sponge filters did work somewhat well using the megasonic technique. Currently the megasonic technique is not quite as effective as the standard technique; however as previously mentioned a notable difference with regards to the levels of shells being present in the samples was seen. Even though only two replicates were carried out for the megasonic compared to three for standard method, there were no damaged cysts observed in the megasonic. It was also noted that the filter sponges in both megasonic and control sponge experiments expanded poorly, which could account for a low recovery of cysts during this experiment compared to the estimated 41-70% recovery expected recovery as noted by UK Environment Agency (2010), on page 82. Personal experience has shown that in *Cryptosporidium* elution from filters, poor filter expansion can impact final recoveries, which could be similar for *Giardia* cysts. This experiment would benefit from being repeated to assess if full sponge expansion would change the results gained.

2.4.5 *A Complete Procedure of Giardia duodenalis Elution using Megasonic Sonication*

Following the data obtained from the work listed above a complete method for filtration of *G. duodenalis* was created. Highlighted areas of difference within this complete method are two megasonic treatments of samples (filter sponge matrix aswell as filter membrane), inclusions of additional wash steps and the lack of a centrifugation step. This aimed to achieve the same benefits as the full megasonic methodology as developed by Kerrouche *et al* (2015) for *Cryptosporidium*, allowing a lower elution volume meaning that centrifugation of the sample can be avoided, which may allow the development of fully automated elution methods. Another benefit is the gentler elution method of megasonic sonication, which appears to allow increased levels of cysts to remain intact throughout the elution process. These benefits highlight the usefulness of megasonic sonication for the elution of *G. duodenalis* cysts from filtration systems. It can be seen from the results (Table 10 and Figure 8) that the standard method is currently more effective at recovering *Giardia* cysts from filters, compared to the megasonic (mean 66 intact cysts vs mean 55 intact cysts, respectively). This was found to be a significant difference using a One-Way ANOVA ($p=0.049$) which shows that the megasonic method still needs further optimised to be comparable to the standard method.

The addition of wash steps appeared to have an impact on the rates of *G. duodenalis* recovery in the three replicates processed (Table 10 and Figure 8) When compared to the previous megasonic methodology used to elute the parasite from the sponge filters the inclusion of wash steps appeared to have created close to a 38% increase in total recovery levels. It was also noted however that the sponges expanded much better during these experiments in comparison to the previous sponge work, which could have contributed to the increased recoveries. As previously mentioned,

Table 10 - Results of Complete Procedure of *G. duodenalis* Elution using Megasonic Sonication

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	74	72	2	72.0%	74.0%	2.7%
	2	73	66	7	66.0%	73.0%	9.6%
	3	64	60	4	60.0%	64.0%	6.3%
	Mean	70	66	4	66.0%	70.3%	6.2%
	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	50	50	0	50.0%	50.0%	0.0%
	2	56	56	0	56.0%	56.0%	0.0%
	3	58	58	0	58.0%	58.0%	0.0%
	Mean	55	55	0	54.7%	54.7%	0.0%

Table 10 - The results of this experiment show that the megasonic methodology still requires optimisation before it can match the recoveries achieved using the standard method of elution (mean number of intact cysts was 55 versus 66, respectively). The recoveries from the megasonic method replicates are however acceptable recoveries and still managed to recover a substantial number of cysts from the sample, further more all of these were found to be intact (mean of 0 shells for megasonic method). This was not the case in the better performing standard method, which had a mean of 4 cyst shells recovered. Where appropriate totals have been rounded down to the closest cyst.

poor expansion of filters has been previously known to limit recovery of *Cryptosporidium*, which could explain the difference in cyst recoveries (if cysts behave similarly) between the first sponge experiments and the complete megasonic results (mean of 23 & 16 cysts (Table 9) vs mean of 66 and 55 cysts (Table 10) for standard and megasonic methods, respectively).

Similarly to the previous experiments, every cyst recovered was seen to be intact and with content when using the megasonic method, compared to the standard method in which some were seen to be damaged (mean of 4 shells in standard method vs 0 shells in megasonic method). This was however not as extreme as in the initial direct

membrane seeding experiment and the level of intact cysts recovered was still higher in the standard method than in the megasonic method. This potentially highlights that levels of damaged cysts resulting from elution may not be as big of a problem when using the standard method as hypothesised, at least in laboratory conditions. This would support comments by Robertson & Lim (2011d) on the subjective possibility of damaged cysts being an issue for the potential of combining *Cryptosporidium* and *Giardia* testing; however an increased number of cysts shells were noted from the standard method which would suggest the megasonic method elution method is kinder to cyst integrity. It would be interesting to assess the efficiency of megasonic elution using field samples. The exposure of cysts to environmental conditions could weaken the cyst shell and cause them to be more likely be damaged during the standard elution protocol, meaning megasonic elution may be a more useful method in this situation.

It should be noted however that gamma irradiated cysts were utilized for this work due to the counted reliability in the numbers of cysts inside the suspensions (100

Figure 8 - Complete Procedure for Megasonic Sonication vs Standard Method

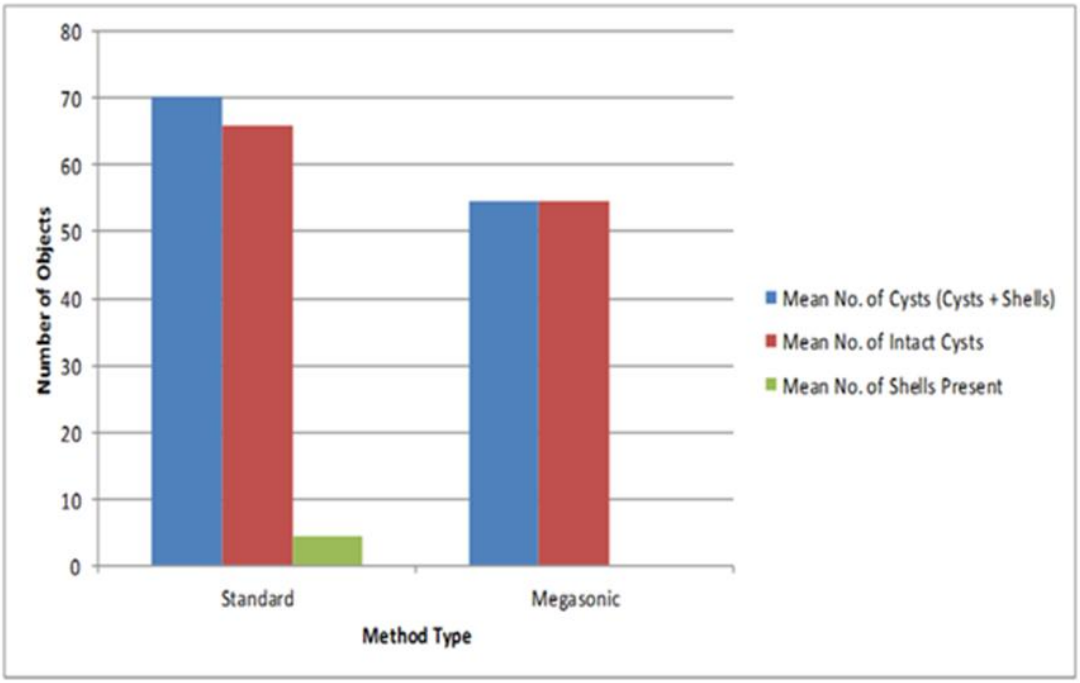


Figure 8 – A graph showing the recoveries found using a complete methodology for megasonic elution against the control standard methodology. It can be seen that the standard methodology achieved higher recoveries of both cysts plus shells (mean of 70 using standard method compared to a mean of 55 using the megasonic method) and also cysts (mean of 66 using standard method compare to a mean of 55 using megasonic method) discounting shells. There were a small number of shells observed within the standard methodology samples which were not seen in the megasonic methodology (mean of 4 shells in the standard method) versus mean of 0 in the megasonic samples).

cysts +/- 2). A difference between gamma-irradiated and live *G. duodenalis* cyst shell dynamics could potentially affect recoveries when compared to other studies using live cysts. On consulting TCS Biosciences, the distributor of the EasySeed cysts in the UK, knowledge of how well the irradiated cysts represent live parasites during filtration is unknown. For this reason both field and laboratory trials using live cysts and megasonic elution would be useful. Previous work has highlighted that the ability of the GC-Combo Dynabead kit for IMS of *Cryptosporidium* and *Giardia* oo/cysts is not affected by the viability of the parasites and so this can be negated as a source of difference between live and inactivated cysts (McCuin *et al*, 2001).

Kerrouche *et al* (2015) achieved comparable recoveries to the standard method when megasonic sonication was employed to elute *Cryptosporidium* oocysts from filters. This shows that it is possible, at least for *Cryptosporidium*, to optimise the megasonic protocol effectively with regards to parasite filter elution; however it is logical to presume that *Giardia* recoveries could be influenced by different factors compared to *Cryptosporidium*, being a different organism. Therefore megasonic sonication could cause difference in recoveries for these organisms if optimisation of the method doesn't alleviate the reduced recoveries observed for *Giardia*.

2.5 Conclusion

In conclusion, the use of megasonic waves to effectively elute parasites, in this case *G. duodenalis*, is still very much in its infancy however it shows promise for use in the future. Currently, recovery levels of megasonic elution have not been found to be as high as in the well optimized, internationally used FiltaMax system. However, the benefits of megasonic elution are not solely found in cyst recovery but in other helpful practical features; reduction in manual labour required to process a filter, the lack of centrifugation required for the method and the seemingly 'kinder' elution process highlight the usefulness of incorporating megasonic waves into the standard elution process (Horton *et al*, Paper awaiting submission: Appendix C).

Development of fully automated systems for recovery of pathogens from water samples is something which should be envisaged for the future of the water industry and the development of methods such as megasonic elution facilitates this process. Automation would mean staff could avoid lengthy manual and repetitive techniques and could potentially enable a higher through-put for water sampling to be carried out by a

water authority. Once optimised to a level in which the recoveries for megasonic elution are at least comparable (if not better) than in the standard FiltaMax methodology, it will be useful to consider megasonic sonication as an option for increased efficiency and reduced manual labour when advancing filtration techniques for the waterborne parasite, *G. duodenalis*. Additionally, the megasonic methodology has a shorter time requirement compared to the standard megasonic process as described by Kerrouche *et al*, 2015).

3. Chapter 3 – Identification of *G. duodenalis* in Environmental Samples in Scotland

3.1 Environmental Contamination of *Giardia duodenalis* within Scotland

Previous work investigating the prevalence of *G. duodenalis* within the environment has often found that levels of the parasite are high, particularly within watersheds worldwide. Animals such as cattle that graze close to these water sources are thought to be the source for the introduction of cysts into these catchments (as reviewed by Plutzer *et al*, 2010). Numerous authors worldwide have investigated the presence of *G. duodenalis* within water catchments of their respective countries, similarly with the levels of infection present within cattle herds (Olson *et al*, 2004; Trout *et al*, 2007; Castro-Hermida *et al*, 2009; Baldursson & Karanis, 2011; Dixon *et al*, 2011; Prystajecky *et al*, 2015). As mentioned in Chapter 1, cattle are thought to serve as a reservoir of various *G. duodenalis* assemblages, potentially shedding up to 19,920 *G. duodenalis* cysts/gram of faeces into the environment (Coklin *et al*, 2010). The animals are thought to both contaminate the environment, as well as water sources local to them. The majority of infections within cattle are thought to belong to assemblage E – the ruminant specific variety of the parasite, with a smaller number reported to be infected with assemblage A (or co-infected with assemblage A and E) which is known to infect humans in some cases (sub-assemblage AII specifically) (Feng & Xiao, 2011).

In Scotland, the most recent large scale study into *G. duodenalis* epidemiology was carried out by Smith *et al* (1993), in which a variety of waters from around the country were tested for the presence of the parasite. Both raw and final waters were tested and the parasite was detected in both water types, with cysts occasionally being seen within final waters following treatment by a water authority. Previous to this a study by Gilmour *et al* (1991) in Scotland found similar results.

This project investigated the presence of *G. duodenalis* in dairy cattle, as opposed to beef. It seems apparent from the literature that there may be a difference in the levels of human infective assemblages found in these two types of cattle, with dairy cattle potentially found to contain more zoonotic assemblages (O'Handley *et al*, 2000; Santin *et al*, 2009; Dixon *et al*, 2011). As dairy cattle will interact with humans more

frequently due to milking, it cannot be overlooked that this may indicate zooanthroponotic transmission to the cattle via humans as opposed to an inherent disposition of zoonotic assemblage infection. Longitudinal studies in other countries carried out have however consistently shown that 100% infection within dairy and beef cattle groups on farms is not uncommon (O'Handley *et al*, 1999; O'Handley *et al*, 2000; Ralston *et al*, 2003).

It would be expected therefore that the cattle samples in this study would follow the same suit, with *G. duodenalis* infections being frequently observed within samples. Intermittent shedding is however common in *G. duodenalis* infection, meaning that all samples from an animal infected with the parasite may not be positive for cyst DNA. Peak shedding has previously been reported to be around 5 weeks from birth in cattle (Ralston *et al*, 2003), which is within the sample set used for this study. The sample set consisted of a total of 26 calves with faecal samples taken at 26 time points throughout January to July 2012.

Previous work by Appelbee *et al* (2003), investigating specifically Canadian beef cattle samples for *G. duodenalis* assemblages, found that the majority of infections were caused by the livestock specific assemblage E. A much smaller proportion was found to be infected with an assemblage A isolate. Their study was similar to the one carried out in this project, as samples were taken from younger animals (2-10 weeks old).

These studies are now historical and thus up-to-date information is desirable. In this chapter, I discuss work which was carried out in an attempt to help fill this lack of up-to-date information on the epidemiology of *G. duodenalis* in Scotland. To do this, DNA extracted from both water samples taken throughout Scotland, as well as a smaller DNA sample set extracted from cattle faeces, will be investigated using 18S-rRNA Polymerase Chain Reaction (PCR) methodologies to ascertain the presence of *G. duodenalis*. Furthermore, within DNA samples positive for *G. duodenalis* DNA, an attempt to identify the specific assemblage to which the parasite belongs will be made. From previous work, as reviewed in chapter one, it would be expected that the majority of infections will belong to assemblage E of the parasite. The level in which assemblage A is present will be of most interest in this work. Previously it has been speculated by health officials that isolates belonging to this assemblage may be to blame for most human cases in Scotland (Pollock *et al*, 2005; Alexander *et al*, 2014). The assemblage

of the parasite is of crucial importance when discerning the threat to public health, as previously discussed. A comparison of the common assemblages found within Scottish Waters to those found within cattle samples from a local area near Edinburgh City, will allow an up-to-date idea of the prevalence of human infective assemblages within Scotland and adds to the global picture for the epidemiology of *G. duodenalis*.

3.2 Materials and Methodologies

3.2.1 *DNA Samples*

Samples of DNA used throughout this work were all supplied from previous studies, totalling around 2000 available environmental samples, from both water catchments and cattle faeces. A range from these samples was selected for analysis. The cattle faecal DNA was extracted for a previous study investigating the presence of *Cryptosporidium* species in young calves from the ages of birth to six months, in 2012 (Thompson, S. PhD Thesis, University of Edinburgh). This project used the cattle samples in a similar way, but investigated them for the presence of *G. duodenalis* DNA instead at various time points. The water samples were taken from a variety of catchments from across Scotland during 2013 by Scottish Water, as per routine testing for *Cryptosporidium spp*. These samples consisted of waste supernatant for discard generated during the Immuno-Magnetic Separation (IMS) (anti-cryptosporidium kit (Wells *et al*, 2015) processing for samples in the water laboratory. Previous work on these samples investigating the presence of *Toxoplasma gondii* DNA had success (Wells *et al*, 2015) and so it was envisaged that the samples could also contain other waterborne parasite DNA. DNA samples were kept at The Moredun Research Institute frozen, thus both sample sets were able to be re-employed in this study in 2015/2016 to investigate them for the presence of *G. duodenalis*.

3.2.2 *DNA Standards (Plasmids)*

A positive control was required to create a standard when assessing the ct value of any potentially positive samples. A set of standard DNA positives were created for use in both qPCR assays. These DNA standards were created using plasmids which had been designed to contain specific DNA targets of *G. duodenalis*. These control plasmids were generated by collaboration between scientists at CRU Wales and The Moredun Research Institute and where made available for this study. For the *G. duodenalis* assemblage wide qPCR the control locus belonged to the livestock assemblage E. This

plasmid amplified locus was termed ‘P15’. β-giardin DNA specific for assemblage A and B was available from plasmids and these were termed ‘G129’ and ‘GDB003’, respectively to be used in the assemblage A and B specific qPCR as controls. To create these controls, a small section of frozen plasmid/glycerol mix (which was kept on ice at all times) was added into a Lysogeny Broth (L-Broth) which contained 10µl of Ampicillin, to prevent non-specific bacterial growth other than bacteria containing the Ampicillin resistant plasmid. The broth was then incubated at 37^{o}C for around 16 hours on a revolving platform to ensure the broth was mixed constantly.

Following this, 2ml of the broth was then transferred into an Eppendorf tube and centrifuged at 8000rpm. Once pelleted the supernatant was removed and an additional 2ml was added and the process repeated. Once deemed that enough bacteria was pelleted, a Qiaprep® Spin Mini Prep Kit was then used to extract the plasmid DNA, as per the manufacturer’s instructions. The DNA was then quantified using a NanoDrop system. A dilution of the DNA was then prepared to appropriate levels for use as controls in the coming qPCR methods (Figure 9).

Figure 9 - An example of Plasmid DNA Standard Curves as Seen in qPCR

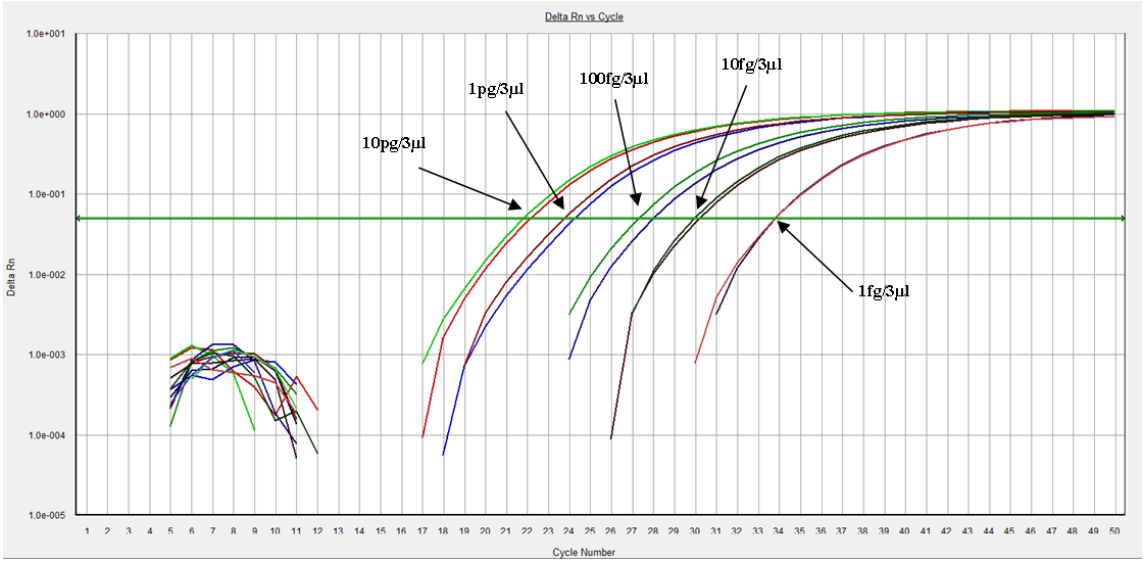


Figure 9 - An example of the plasmid DNA standard curves as seen in each qPCR result. DNA was diluted ten fold in each case, allowing a series of positive control curves to be seen in each experiment. Standard curve DNA dilutions were set at 10pg/3µl, 1pg/3µl, 100fg/3µl,10fg/3µl and at 1fg/3µl. Previous tests found that decreasing the concentration to less than 1fg/3µl resulted in frequent inconsistent curves using 100ag/3µl etc. The DNA curves ranged from a value of 22CT (10pg/3µl) to 34CT (1fg/3µl) as can be seen in the above figure.

Figure 10 - An Example of Internal Control Within Samples

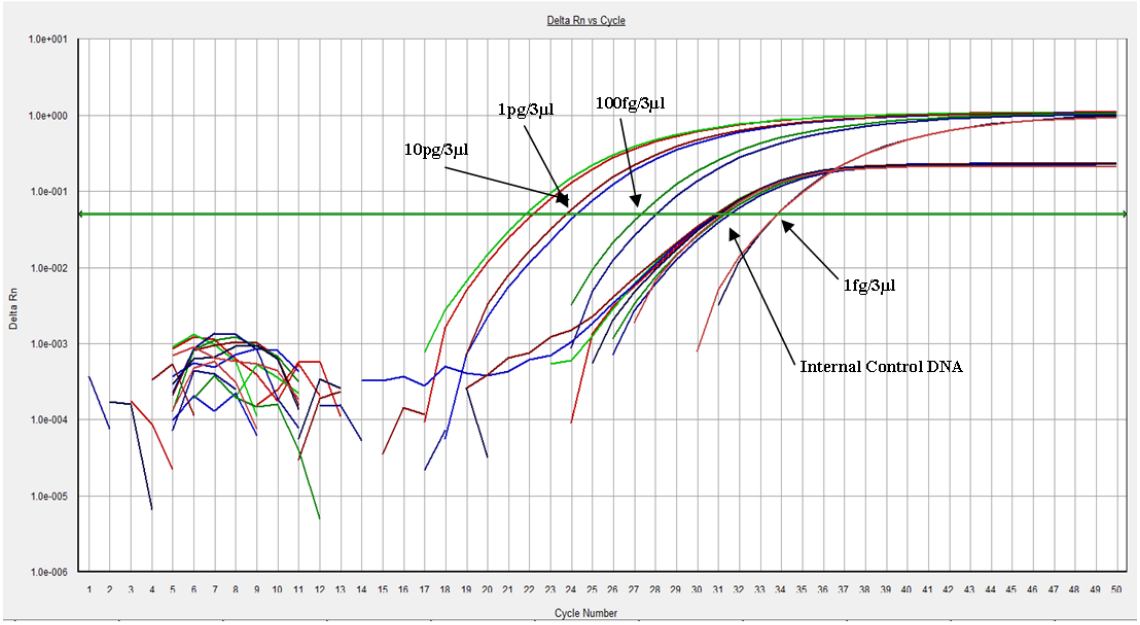


Figure 10 - An example of the internal control values observed when it was included in samples. In this example the internal control was included within the plasmid DNA (DNA dilution of 10fg/3µl not included in this picture for clarity, as this appears close to the CT seen for internal control DNA) standards. As can be seen the internal control DNA has a CT value of between 31-32. The presence of the internal control DNA highlighted that there was no inhibition within the various DNA standards. Internal control DNA was seen at a similar CT value when using real samples and it inhibition was never seen or thought to be present in samples used in this work using this internal control.

3.2.3 *PCR (qPCR and n-PCR) Methods*

The work in this project was initially based on multiplex qPCR (quantitative Polymerase Chain Reaction) techniques which were developed and validated at the Cryptosporidium Reference Unit, Wales (Elwin *et al*, 2014). From this work two multiplex qPCR assays were developed for the detection of *G. duodenalis* DNA. The first was designed as an assemblage wide qPCR, being able to detect a variety of *G. duodenalis* assemblages in samples. The second was designed to be specific in detecting *G. duodenalis* assemblage A and B DNA within a sample. Both would be used in combination to gain insight into the presence of *G. duodenalis* within a sample where possible.

Positive samples detected using the assemblage wide qPCR, will be confirmed as positive using a nested-PCR based on the work by Appelbee *et al* (2003) targeting the 18S-rRNA locus. Suitable positive DNA would then be sent for sequencing allowing an idea of the specific assemblage present within the sample. These samples which had been detected using the initial qPCR and n-PCR would then be investigated using the

assemblage A and B specific qPCR developed to further confirm the presence of zoonotic assemblages, either A or B (Elwin *et al*, 2014). Notably this method would be useful on samples which were positive for *G. duodenalis* DNA using the initial qPCR and n-PCR. Sequencing may confirm the presence of assemblage E within samples, as would be expected, however if an assemblage A isolate was also present (mixed infection) this could be highlighted by the A and B specific qPCR. In doing this a picture of the spread of assemblages throughout samples can be obtained.

Giardia Assemblage Wide Beta-Giardin qPCR

The first technique was designed to detect any *G. duodenalis* assemblages within the sample by targeting the Beta giardin (β-giardin) gene. This gene is unique to the *Giardia* parasite and present in all assemblages of *G. duodenalis*. The qPCR has been validated by Elwin *et al* (2014) to detect DNA of assemblages A, B, D and E; detection of assemblages C and F is also possible based on *in silico* analysis. Primers and probes were purchased and created by Eurofins Genomics (MWG), primers were delivered lyophilised and were re-constituted using 1xTE buffer before use. The 1xTE buffer was made by adding 400µl of 0.5M TRIS Buffer (Tris(hydroxymethyl)aminomethane) and 40µl of 0.5M EDTA (Ethylenediaminetetraacetic acid) to 199.5 mls of ultrapure water. The solution was then autoclaved for sterility before use.

In this assemblage wide qPCR the forward primer used, termed 'BGF2', had the sequence of 5'-GAGGCCCTCAAGAGCCTGAA-3'. The reverse primer, termed 'BG2R2', had the sequence of 5'-CTCGACGAGCTTCGTGTT-3'. Both primers were diluted to a concentration of 10µM for use in the qPCR. The fluorogenic probe used in the qPCR was termed 'BGT2' and had the sequence 5'-VIC-ATCGAGAAGGAGACGATCGC-MGB-NFQ-3'. At the 5' end of the probe a fluorescent dye, 'VIC' (excitation wavelength 530, emission wavelength 549), was attached followed by a minor groove binder molecule (MGB) and a non-fluorescent quencher (NFQ) on the 3' end. The probe was also diluted down to 10µM before use. The PCR master mix (Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0) used was purchased from Thermo-fisher. An internal control was also added when deemed appropriate to ensure that no inhibition occurred within the qPCR originating from the DNA samples to be tested. This internal control was purchased from

Primerdesign Ltd and came as a kit containing both the internal control DNA and a probe/primer pre-prepared mix.

The primer/probe mix was re-constituted using ultrapure water before use, as it was lyophilised on arrival, prepared according to the manufacturer's instructions. Internal control DNA was diluted to 1:10 before use as specified on the assay protocol developed by Elwin *et al* (2014). The internal control probe read through a different fluorescent channel, CY5 (excitation wavelength 649, emission wavelength 670) to that of the VIC probe used in the qPCR for samples, meaning that there would be no interference with detection between controls and sample. The internal control was only included until satisfied that samples were not affected by inhibition.

Table 11 - Beta-Giardin qPCR Detection Assay Components and Volumes

Component	Specific Reagent Used	Volume of Reagent Per Reaction (µl)
PCR master mix	ABI Environmental Master Mix 2.0	12.5
Forward Primer (10µM)	BGF2 (GAGGCCCTCAAGAGCCTGAA)	0.75
Reverse primer (10µM)	BG2R2 (CTCGACGAGCTTCGTGTT)	1.5
Probe (10µM)	BGT2 (VIC-ATCGAGAAGGAGACGATCGC-MGB-NFQ)	0.25
Internal DNA Control (1:10 Dilution)	Primerdesign Internal Control	2.5
Internal Control Primers & Probe	Primerdesign Internal Control	1
Ultrapure Water	Nuclease free / molecular grade	3.5
Template DNA	Sample DNA to be tested	3
	Total Volume	**25**

Table 11 - This table show the various components of the qPCR assay designed by CRU Wales to detect various *G. duodenalis* assemblages within a DNA sample. Total volumes within the assay were each 25µl. An internal control was included within the samples to detect if any inhibition occurred within the samples which could have caused false-negative results. If the internal control achieved a result as expected (CT value of 26+/-3 as set by manufacturer with neat DNA, therefore expected to be a higher CT value for diluted control DNA), negative samples could be trusted as being negative. If the internal control was not to be included in a sample then the components were replaced with additional ultrapure water instead to maintain a 25µl total volume in all reactions. Table based on assay developed by Elwin *et al* (2014).

Table 11 illustrates these details along with specific volumes required for each reaction in the assay. Experiments were run on an Applied Biosystems™ 7500/7500 Fast Real-Time PCR System. The thermo-cycling programme developed was as follows, as validated by CRU Wales: Hold (Hot Start) at 95^{o}C for 600 seconds, followed by 50 cycles of 95^{o}C for 15 seconds and annealing 60^{o}C for 60 seconds. Data was specified to be collected from the red (CY5) and yellow (VIC) fluorophore channels during each annealing/extension phase (60^{o}C step of each cycle). When reading the results a fluorescence threshold was set at 0.05. Each sample to be tested using this qPCR was carried out in duplicate for greater sensitivity.

18S-rRNA Nested-PCR

This assay was based on work by Appelbee *et al* (2003) and the methodologies and set up were identical apart from a change in the PCR buffer used and the annealing temperature of both amplification rounds. The annealing temperature was decreased to 45^{o}C in both rounds of the n-PCR, as opposed to 55^{o}C and 59^{o}C (for first and second rounds of PCR, respectively) as suggested by the authors. The lowered settings yielded clearer results compared to the initial set temperatures. The n-PCR consisted of two stages of amplification of the locus which may be present in the sample (Figure 11). Initially the first PCR amplified a 479bp product within the 18S-rRNA ribosomal unit, using the forward and reverse primers, Gia2029 (5'-AAGTGTGGTGCAGACGGACTC-3') and Gia2150 (5'-CTGCTGCCGTCCTTGGATGT-3'), respectively, from 2µl of sample DNA. This product was then diluted in 120µl of ultrapure water, before 1µl of diluted product was taken to be used in the second round PCR. The second round of the PCR then amplified a 292 bp fragment within the same locus from the first round amplified product. The second round used the forward and reverse primers of RH11 (5'-CATCCGGTCGATCCTGCC-3') and RH4 (5'-AGTCGAACCCTGATTCTCCGCCAGG-3'), respetvely. All four primers were purchased from Eurofin Genomics (MWG) and reconstituted in ultrapure water. In the first round PCR reactions, each consisted of the following: 1.25µl (at a concentration of 10pM/µl) of each appropriate primer, 2µl of 10x in-house PCR buffer (Katzer *et al* , 2014) 13.35µl of ultrapure water and 0.15µl of *Taq* polymerase (Qiagen). 2µl of sample DNA was then added into the reaction, totalling 20µl per reaction. The second round reactions were similar, however only 1µl of first round product was added and so

additional 1µl of ultrapure water was added (totalling 14.35µl) to total 20µl per reaction (Table 12).

Table 12 - 18S rRNA n-PCR Detection Assay Components and Volumes

Component	Specific Reagent Used	Volume of Reagent Per Reaction (µl)
10x In-House PCR Buffer	The Moredun Research Institute	2.00
R1: Forward Primer (10pM/µl)	Gia2029 (AAGTGTGGTGCAGACGGACTC)	1.25
R1: Reverse primer (10pM/µl)	Gia2150 (CTGCTGCCGTCCTTGGATGT)	1.25
R2: Forward Primer (10pM/µl)	RH11 (CATCCGGTCGATCCTGCC)	1.25
R2: Reverse primer (10pM/µl)	RH4 (AGTCGAACCCTGATTCTCCGCCAGG)	1.25
Ultrapure Water	Nuclease free / molecular grade	***R1:*** 13.35 / ***R2:*** 14.35
Taq Polymerase	*Taq* Polymerase	0.15
Template DNA	Sample DNA to be tested	***R1***: 2 / ***R2***: 1
	Total Volume Each Round	**20**

Table 12 - This table lists the various components and volumes of the n-PCR assay adapted from the one developed by Appelbee *et al* (2003). The assay consisted of two rounds, with differences in volumes being listed as for "***R1***" if used in the first amplification round, or "***R2***" if used in the second. The first round used the two primers termed "Gia2029" and "Gia2150", whilst the second used the two termed "RH11" and "RH4". Differences in the volume of ultrapure water added were due to a smaller volume of diluted first round product being required for the second round setup.

Samples were processed in triplicate for greater sensitivity within the PCR. The thermal conditions for each round of the n-PCR were identical to eachother: Hold (Hot Start) at 94^{o}C for 300 seconds, followed by 35 cycles of 94^{o}C for 45 seconds, annealing temperature of 45^{o}C for 45 seconds and 72^{o}C for 45 seconds. Following this the products were ran on a 2% Agarose Gel (4g Agarose powder, 200ml 1xTAE and 12µl GelRed) at 110V for 45 minutes and viewed using ultraviolet light. Confirmation of bands at around 292 bp allowed positive identification of *G. duodenalis* DNA within the samples.

From the n-PCR, samples which displayed clear bands with little interference will be selected for sequencing. These samples will be cleaned using a Wizard® SV Gel + PCR Clean-Up System as per the manufacturers instructions, their DNA concentration

determined using a NanoDrop System and then diluted and sent for sequencing in both directions. Sequences were then compared to determine the assemblage of the sample DNA.

Figure 11 - Nested PCR of 18S-rRNA Ribosomal Unit

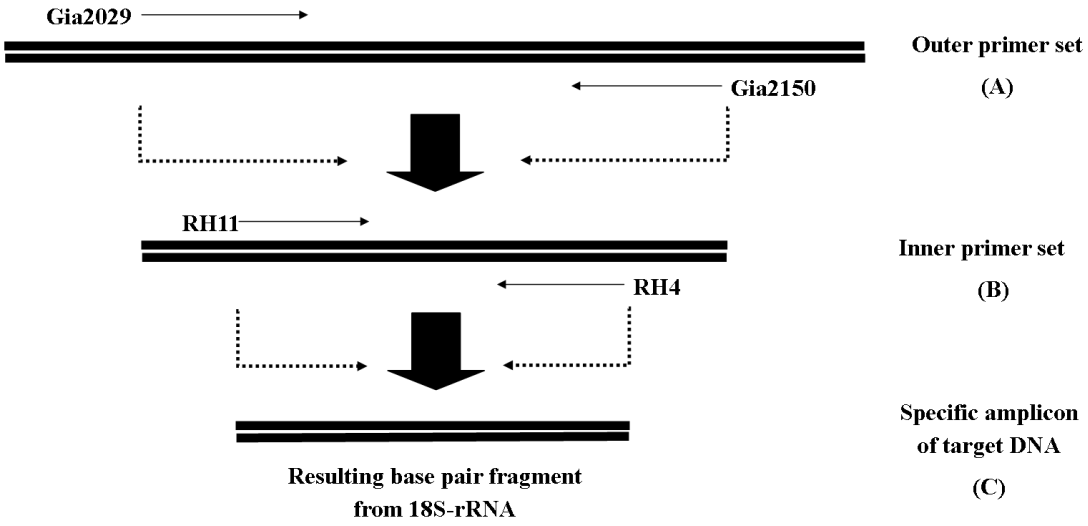


Figure 11 - This figure shows a visual representation of the basic process of the nested Polymerase Chain Reaction (n-PCR) technique used within this work. To begin a set of 'outer' primers were used to amplify a specific product size within the 18S-rRNA Ribosomal unit locus (A). A second set of 'inner' primers were then used to further amplify a smaller sized product from within the resulting product amplified during the first sequence of PCR (B). This process then resulted in a final smaller sized product being amplified for detection, if present within the sample being tested (C).

Giardia Assemblage A + B Tpi qPCR

A positive result by both the assemblage specific qPCR and nested PCR in a sample will be investigated for the presence of *G. duodenalis* assemblages (A and B). These two assemblages are important as previously covered due to their potential for zoonotic transmission and human infection. All reagents were purchased/used were identical to the above section, aside from primers and the probes. In the *G. duodenalis* assemblage A and B specific assay, the assay contained four primers as opposed to two (each assemblage had a forward and reverse primer set), which were purchases from Eurofins Genomics (MWG). This qPCR method targeted the trio-phosphate isomerase (tpi) gene, allowing specific detection of only assemblages A or B DNA of *G. duodenalis* within a sample.

For assemblage A, the forward and reverse primers were termed 'AF' and 'AR' respectively. The 'AF' primer had the sequence of 5'-CATTGCCCCTTCCGCC-3', while 'AR' had a sequence of 5'-CTGCGCTGCTATCCTCAACTG-3'. The probe for assemblage A was termed 'AT' and had a sequence of 5'-FAM-CCATTGCGGCAAACA-MGB-NFQ-3'. The probe also had the fluorescent dye

'FAM' (excitation 495, emission 520) located on the 5' end of the sequence, along with a minor groove binder molecule (MGB) and a non-fluorescent quencher (NFQ) located on the 3' end.

The assemblage B forward and reverse primers were termed 'BF' and 'BR', respectively. The 'BF' primer had the sequence 5'-GATGAACGCAAGGCCAATAA-3', while 'BR' had the sequence 5'-TCTTTGATTCTCCAATCTCCTTCTT-3'. The probe for the assemblage B specific assay, termed 'BT', had a fluorescent dye 'VIC' located on the 3' end of the sequence with again a minor groove binder molecule (MGB) and a non-fluorescent quencher (NFQ) located on the 5' end. The sequence for

Table 13 - Tpi qPCR Detection Assay Components and Volumes

Component	Specific Reagent Used	Volume of Reagent Per Reaction (µl)
PCR master mix	ABI Environmental Master Mix 2.0	12.5
Assemblage A Forward Primer (10µM)	AF (CATTGCCCCTTCCGCC)	0.75
Assemblage A Reverse primer (10µM)	AR (CTGCGCTGCTATCCTCAACTG)	2.25
Assemblage A Probe (10µM)	AT (FAM-CCATTGCGGCAAACA-MGB-NQF)	0.25
Assemblage B Forward Primer (10µM)	BF (GATGAACGCAAGGCCAATAA)	0.75
Assemblage B Reverse Primer (10µM)	BR (TCTTTGATTCTCCAATCTCCTTCTT)	2.25
Assemblage B Probe (10µM)	BT (VIC-AATATTGCTCAGCTCGAG-MGB-NFQ)	0.25
Internal DNA Control (1:5 Dilution)	Primerdesign Internal Control	1
Internal Control Primers & Probe	Primerdesign Internal Control	1
Ultrapure Water	Nuclease free / molecular grade	1
Template DNA	Sample DNA to be tested	3
	Total Volume	**25**

Table 13 - This table show the various components of the qPCR assay designed by CRU Wales to detect assemblages A and B of *G. duodenalis* within a DNA sample. Total volumes within the assay were each 25µl. An internal control was included within the samples to identical to the previous assemblage wide assay, however here the internal control DNA dilution was reduced. If the internal control achieved a result as expected (CT value of 26+/-3 as set by manufacturer with neat DNA, therefore expected to be a higher CT value for diluted control DNA), negative samples could be trusted as negative. If the internal control was not to be included in a sample then the components were replaced with additional ultrapure water instead to maintain a 25µl total volume in all reactions. Table based on assay developed by Elwin *et al* (2014).

this probe was 5'-VIC-AATATTGCTCAGCTCGAG-MGB-NFQ-3'.

The same internal control was used identically to previous in this assay; however its dilution factor was reduced (down to 1:5). Thermal Cycler program conditions in this assay were identical to those used in the assemblage wide qPCR, however data collection in each annealing/extension phase (60^{o}C step of each cycle) was collected from the red (CY5), yellow (VIC) and green (FAM) fluorophores channels. Identical to in the previous assay, when reading the results of the assay a fluorescence threshold of 0.05 was set. Table 13 illustrates these details along with specific volumes required for each reaction in the assay for assemblage A + B identification. Each sample to be tested using this qPCR was carried out in duplicate for greater sensitivity.

3.3 Results and Discussion of Environmental DNA Screening

3.3.1 *Detection of G. duodenalis in Scottish Water*

The United Kingdom is well known for frequent wet weather, Scotland in particular. The levels of water in the environment and frequency of catchements in the natural environment mean that waterborne giardiasis is a potential threat to the public. Animals will also become infected and further contaminate areas with cysts; with has further links of human infection as discussed in Chapter 1. Investigation into the presence of *G. duodenalis* cysts in Scottish Waters will allow an idea of the threat of the parasite to the public through water catchments.

The results of the water sample DNA, which consisted of water supernatant taken from the end stage of Scottish Water IMS sample processing for *Cryptosporidium*, was disappointing in terms of positive identification of cyst presence, but was encourgaging in terms of lack of environmental comtamination. Samples were screened using the assemblage-wide qPCR (Table 10) to identify the presence of samples positive for *G. duodenalis* DNA. Each assay also contained plasmid DNA at varying dilutions as positive controls (Figure 9).

Within each of the samples the internal control amplified as expected suggesting a true negative sampe in each case, with no inhibition (Figure 10). After testing about 200 samples it was decided not to continue to screen all water samples as only two of them (1399: Tomnavo C-121113 and 1407: Tomnavo C-171113) were potentially positive, with 1 out of 2 replicates weakly positive in each sample (Figure 12). These

two positive samples were from the same location at two different time points (Appendix A), 12th November 2013 and 17th November 2013, for samples 1399 and 1407, respectively. This information could suggest that level of cysts in the raw waters here is greater than in the others which were tested, however due to the low numbers of positive samples, combined with the fact that the samples were not definitively positive in both replicates; this cannot be disconcened and is speculative.

Figure 12 - Potentially Positive Water sample 1399 and Positive Control Standard Curves

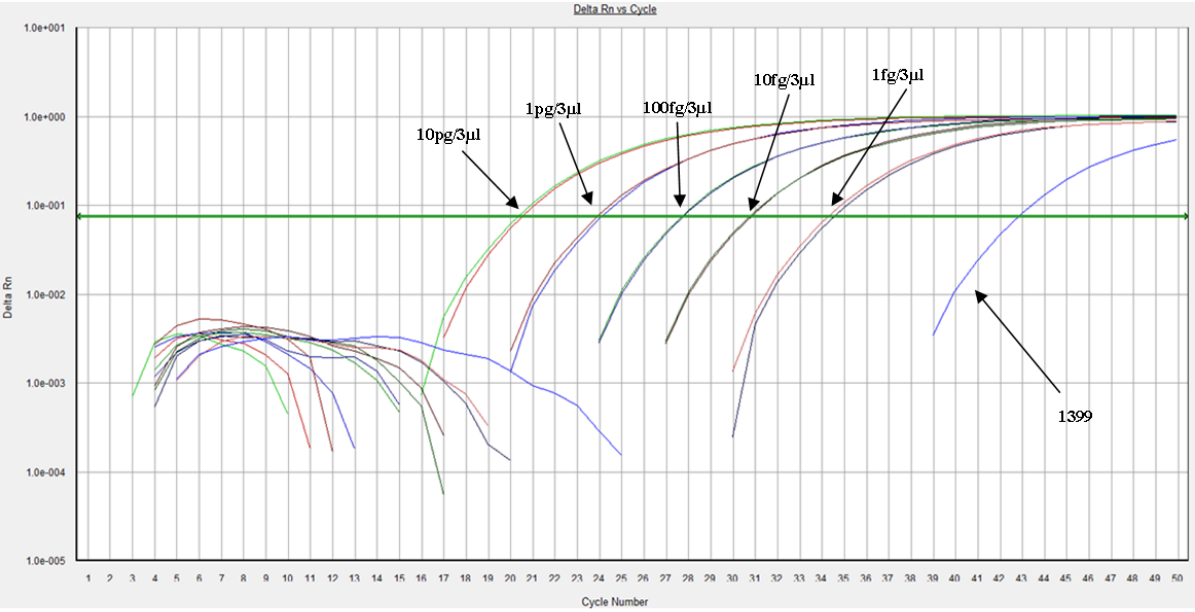


Figure 12 - This figure shows the results of one of the two potentially positive samples found within the Scottish Water samples. Sample number 1399 was found to have one out of two replicates very weakly positive. As the CT value is high however (43), it cannot be assumed that this sample is actually positive. The figure also shows the plasmid DNA standards and their concentrations and relative CT values, ranging from CT value of ~20.5 to 34.5.

The internal control amplified at a slightly higher CT value (31-32) than suggested by the manufacturers (26+/-3), this was due to the 1:10 diltuion as per the CRU protocol and so still functioned as expected. Only 2 out of over two hundred samples, were detected as being potentially positive (sample 1399 and 1409), with 1 out of 2 replicates being weakly positive; each having a CT value of above 40 (Figure 12). Samples identified as positive originated from the same catchment at two different time points (1399 – 12th November 2013, 1407 – 17th November 2013). As there were no definitively positive samples, neither the n-PCR nor assemblage A and B specific qPCR were used on any of the Scottish Water samples.

The reasoning behind the lack of positive samples is unknown. Going by the results found within this study, evidence suggests that the water samples contained no *G. duodenalis* DNA within them. This is however somewhat unexpected as previous

studies, Gilmour *et al* (1991) and Smith *et al* (1993), found evidence of the parasite within Scottish Waters (both raw and finished waters). It would have been expect that, even if the contamination levels had decreased within Scotland from this time (which is very unlikely for raw waters), then a proportion of samples would still be detected as positive for the *Giardia* DNA.

The samples, on which this study was based, were derived from Scottish Water post-IMS waste liquid from routine *Cryptosporidium* screening. The nature of the samples could have therefore had an impact on the presence of DNA from other organisms within the sample. There is a possibility that *G. duodenalis* cysts coud have been lost during the processing, especially if these were in small numbers within the sample to begin with through routine processing of the sample. The waste from the IMS which would later become the samples used in this study, along with others which utilized them (Wells *et al*, 2015), would have been stored routinely for 1-2 weeks before they were processed further. This time delay could have been detrimental to *G. duodenalis* cyst integrity and DNA composisition, as they would have already been through various stages of processing. Following this period: post IMS samples were centrifuged twice and washed inbetween with TE buffer and vortexed vigorously for 30 seconds as part of a wash process (Wells *et al*, 2015). These factors alone, not considering the processing required before the IMS stage was reached by Scottish Water (filtration of water sources etc) could have all impacted (both alone or in combination) in a way which was detrimental to cyst detection within this study. Furthermore the fact that two samples were detected to be potentially weakly positive would suggest that the parasite DNA could be present, however in very low quantities within the sample.

The study carried out Wells *et al* (2015) did not share these difficulties, achieving positive results for *Toxoplasma gondii* DNA within these samples. This parasite is also a waterborne pathogen much like *G. duodenalis*. The absence of *G. duodenalis* DNA is therefore unexpected, as the oocysts of *T. gondii* are not much larger than a *G. duodenalis* cyst (10-12µm (Dubey *et al*, 1970)) and therefore if present, the cysts would have been trapped similarly to the oocysts within the filter on sampling. Despite their size being somewhat similar, the two parasites however are known to be different in terms of stability (Dubey, 1998; Olson *et al*, 1999), which could be a factor in the identification of *T.gondii* often, but absence of *G. duodenalis*. Due to this, degradation could have likey resulted in the unexpected negative results which were found within this study when investigating the water samples. *G. duodenalis* is less robust when

experiencing environmental stress when compared to that of *Cryptosporidium* species, for example (Olson *et al*, 1999). Furthermore, as described in Chapter 2 of this thesis, *G. duodenalis* has been seen to become destroyed or damaged much more often during filtration/elution processes when compared to that involving *Cryptosporidium*. This could also have resulted in a percentage of parasite recovered within the filter being lost throughout processing at an increased rate compared to that of *Cryptosporidum*. This would have initially limited the levels of *G. duodenalis* cysts within the samples to begin with, which when followed by further losses throughout processing, may have reduced parasite DNA levels to below the range detectable by the q-PCR method employed in this work. Both *Toxoplasma gondii* and *Cryptosporidium parvum* are known to form hardier environmental parasite forms (oocysts) compared to that of *G. duodenalis*, which would explain why *Cryptosporidium* is able to be detected in this manner by water authorities, along with the results for *T. gondii* found by Wells *et al* (2015).

The sites in which high detection levels of *Toxoplasma gondii* were found within the study by Wells *et al* (2015) were negative for *G. duodenalis* presence in this study (samples 1384-1418). The *T. gondii* positive samples were in the same overall catchment area as the two *G. duodenalis* potentially positive samples in this study (Tomnavoulin), however from different sites; the samples potentially positive for *G. duodenalis* in this study were negative for *T. gondii* in the study by Wells *et al* (2015).

Further work would be greatly benefitial within this area, as water is known to be a huge factor in human infection with *G. duodenalis*. Specifically, it would be useful to screen the entirety of samples to enable detection of additional positive results. These could then be specifically investigated using further techniques, which may be more sensitive, to further confirm positive identification of DNA within them. Unfortunately due to the time limitations during this project, this was not possible and therefore the Scottish Water samples were not investigated any further upon the low numbers of positives found detected. Judging by the trend however, in data which was gained from these samples, it would be thought that positive detection of *G. duodenalis* within samples would be rare.

Moving forward, I believe that an additional study based on the techniques utilized within this one, with a different sample set taken specifically for the purpose of the identification of *G. duodenalis*, would most likely have a different outcome.

Previous work within the Scottish environment shows that *G. duodenalis* is frequently found within water catchments, which did not agree with the findings within this study. This unfortunately means that the true up-to-date knowledge of *G. duodenalis* within Scottish Waters still eludes us, requiring further work and specifically designed studies to reveal the true level of contamination, along with the potential threat to the Scottish public.

3.3.2 *Cattle Faecal DNA Sample Results*

Assemblage Wide qPCR

Screening bovine faecal DNA samples had much more success, identifying samples in which *G. duodenalis* was present, compared to the previous Scottish Water investigations. Although not all bovine samples were tested for the presence of *G. duodenalis*, a substantial amount was found to be positive for the DNA using the assemblage wide β-Giardin qPCR (Appendix B). In total 16 / 25 calves were found to be shedding *G. duodenalis* at somepoint during the time period investigated. A single calf (ID 2412) was not screened for the presence of *G. duodenalis* DNA, as only few of its samples were located. The remainder of the calves (9), when tested, were not found

Figure 13 - An Example of Samples Positive for *G. duodenalis* using an Assemblage-wide qPCR

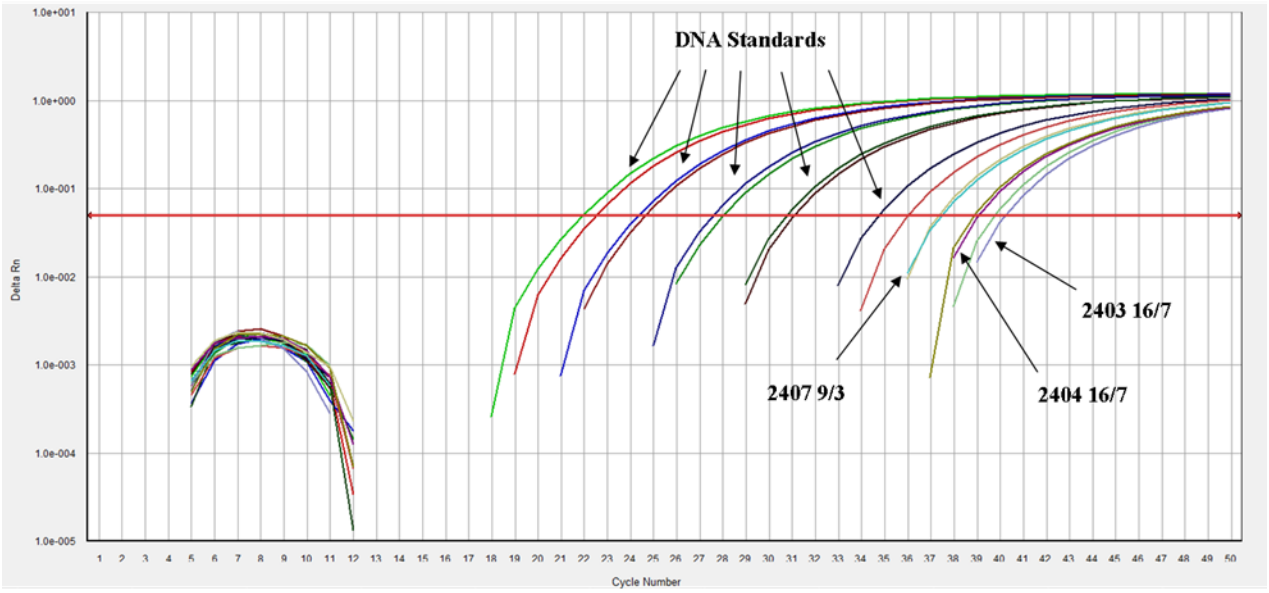


Figure 13 - This figure shows an example of three positive samples for the detection of *G. duodenalis* DNA. Positive DNA samples featured in this example are animal 2403, sample date 16th July; animal 2404, sample date 16th July and animal 2407, sample date 9th March. DNA standards were also included within each qPCR reaction and are highlighted on this figure (although not labelled here - for DNA standard labelling see Figure 9.). Samples were ran in duplicate and within both replicates of each of these samples, *G. duodenalis* DNA was detected. This was not however always the case, with some samples only being positive in one of two replicates.

to be positive for the presence of *G. duodenalis* DNA within their faeces. Sample processing was prioritized to be around 5 weeks from birth for each animal, as this was noted from literature to be the expected time for *G. duodenalis* shedding in the animals (Ralston *et al*, 2003). Often when positive by the assemblage specific β-Giardin qPCR, CT values would be higher than those for the control standards. This appeared consistently throughout the samples, however the CT values were high enough to be deemed positive using this qPCR method (Figure 13).

18S-rRNA n-PCR Results

As numerous animals were found to be positive these were then taken forward to be confirmed for the presence of *G. duodenalis* DNA. 28 samples in total were analysed using the 18S-rRNA n-PCR based on the methods by Appelbee (2003). Initially samples were analysed using the initial reaction conditions set by the authors, however the assay failed to amplify the locus using an annealing temperature of 55^{o}C and 59^{o}C for first and second rounds of PCR, respectively. This was then trialed at 45^{o}C and 50^{o}C

Figure 14 - Example of Results of 18S-rRNA n-PCR Used on Samples Detected as Positive for *G. duodenalis* DNA

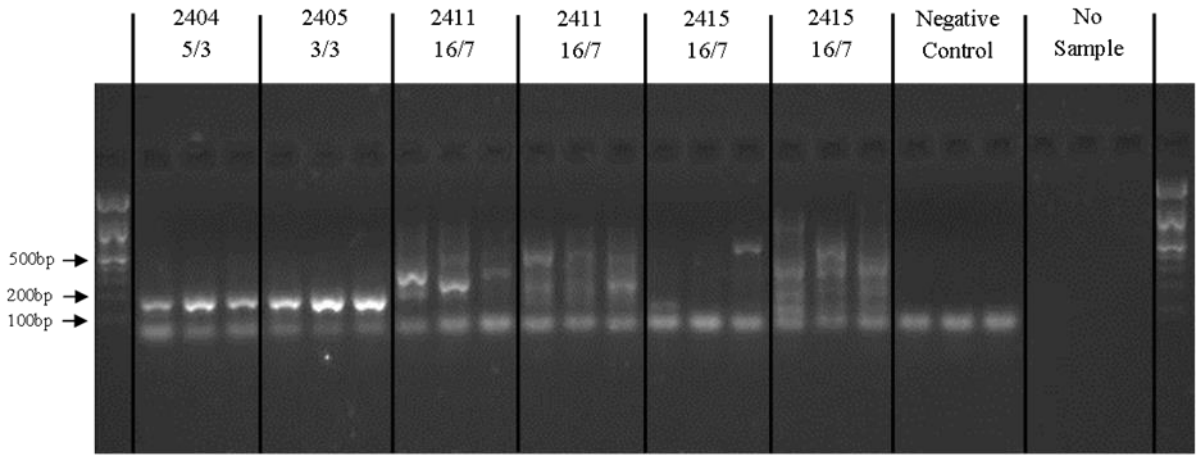


Figure 14 - This figure shows a result from the failed n-PCR which aimed to amplify a 292bp region of the 18S-rRNA locus within positive *G. duodenalis* samples. It can be seen that the amplified product was not the correct size and instead appeared to be around ~190bp instead. This was thought to be due to a change in annealing temperatures which was required as initially the n-PCR did not work using the suggested conditions. The negative control was not amplified as expected, meaning that the assay was free from contamination. Samples 2411 and 2415 look to have increased non-specific amplification within them, however there are also products weakly amplified at around what could be 292bp. This is especially noticeable within replicate 1 of sample 2411. Optimisation nor repeats of this work were unfortunately not possible due to time limitations.

(identical for both rounds) in an attempt to correct the issues causing the assay to fail within this study. Both temperatures yielded amplified product, with 45^{o}C creating a

cleaner version compared to the 50^{o}C. Lowering annealing temperature does however increase the chances of non-specific amplification, which could mean that the region to be amplified (292bp product) could be missed. Samples were processed using the annealing temperature of 45^{o}C, but unfortunately an incorrect region of ~190bp was amplified instead of ~292bp as expected, meaning that the n-PCR did not allow confirmation of *G. duodenalis* DNA presence. Further work optimizing this assay would have allowed eventual amplification of the correct region, however due to the time limitations within this part of the work this was only able to be attempted briefly. A temperature gradient with a control of *G. duodenalis* DNA would have allowed this assay to be optimised for both correct region amplification, as well as lowering the levels of non-specific amplification which were present.

Figure 14 shows an example of an unsuccessful result found by using this assay with the conditions described above. Amplicons of the correct size were to be originally sent for sequencing, however as the samples did not amplify the correct product this was not possible. Sequencing data would therefore not be available to identify whether the assemblage of *G. duodenalis* within these positive samples was E, A or B.

Assemblage A and B Specific qPCR Results

This part of the study was planned to be origionally a secondary check to confirm the potential presence of assemblage A or B following on from successful confirmation via the n-PCR. Furthermore this qPCR would be useful in detection mixed assemblage infections which is often seen with assemblage A and E (see Chapter 1), which may have been highlighted from sequencing had it been possible.

This qPCR was also unable to be employed for this use due to problems achieving amplification. Plasmid DNA controls for both assemblages A and B were trialed within the assay however amplification of these targets did not occur. It is unknown as to the reasoning behind why this qPCR did not work as expected, however further optimisation will be required. Unfortunately due to time limiations within this study this work could not be completed.

Cattle Age Association with Infection of G. duodenalis

Although it was disapointing that the exact assemblage to which the parasite belonged within each positive sample could not be identified, insight into the prevelance of *G. duodenalis* within cattle was still evaluated. Data gathered from this study in terms of positive samples was plotted against the age of animals within the study, as can be seen in Figure 15. Peak infection within the samples appeared to be around week 4 of calf life, with the majority of positive infections occurring during this time. Infections appeared around this time period, raising from week 2, peaking at week 4 before droping off again until 7 weeks of age (Figure 15).

Figure 15- Association of Age with Detection of *G. duodenalis* Infection

Week No.	Total Number of Samples (n)	Positive Samples	Negative Samples	Percentage of Positive to Negative
1 (1-7 Days)	18	0	18	0%
2 (8-14 Days)	21	1	20	5%
3 (15-21 Days)	21	5	16	24%
4 (22-28 Days)	34	10	24	29%
5 (29-35 Days)	31	6	25	19%
6 (36-42 Days)	32	4	28	13%
7 (43 - 49 Days)	7	2	5	29%
8 (50-56 Days)	0	0	0	0%
Totals	**164**	**28**	**136**	**17%**

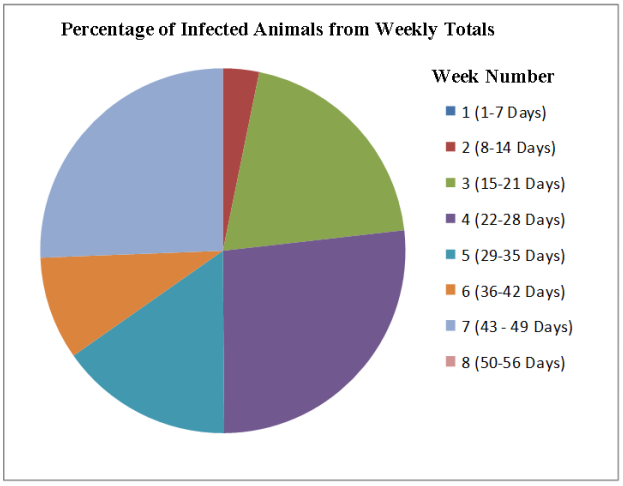


Figure 15 - This figure shows the association of age (in weeks) with the detection of *G. duodenalis* infection within calves. It was found that the majority of infections took place within the periods of between 3-6 weeks of age in the animals within this study. Week 7 also had a large percentage of infections, however as the numbers of samples here were lower compared to other weeks, these are though to be less accurate. Peak infection detection appeared to be around week 4, with numbers falling either side of this date. Samples within week 1 were not detected as being positive for *G. duodenalis* DNA. There was similarly no *G. duodenalis* DNA detected in samples within week 8. Data from weeks 1 and 8 were included in the chart, however the numbers of positive samples were all 0.

Samples within week 4 had the largest numbers of infection (42%), which was followed closely by week 7 (40%). As only a small number of samples from week 7 were taken this higher number of animals found to be infected could be untrue. The infection pattern with samples around weeks 3-5 appears consistent with literature, which tends to report peak shedding within week 5 for infected calves. Samples were also taken at around 6 months of age, and in these samples (Appendix B) it can be seen that interestingly animals which were born first were, all negative for the detection of *G. duodenalis* DNA at this point in time. Animal which were born later were found to be

often positive at 6 months of age. These results do not suggest that the animals which were born first clear the infection by this time, as shedding is known to be intermittent. Increased numbers of samples within this 6 month period would have been useful to overcome this, which may have lead to detectable *G. duodenalis* DNA at this tine period. It could mean however that by this time period the animals which were born and therefore infected, before others, were shedding lower numbers of cysts at this 6 month time point, which may not have been often detectable by the qPCR. It is known that the shedding profile of *G. duodenalis* in cattle is often seen from weeks 2-10, which would be somewhat comparable to this studies finding. Animals which were born last within this study could still be shedding higher levels at 6 months, compared to those born at the beginning, which could be why increased infections was seen later within them. Some animals also had samples taken within the month of May, which were all found to be negative for *G. duodenalis* DNA when tested. This further highlights the sporadic shedding of the parasite in hosts, as the samples within July had some positive results. Again, further sampling within the month of May would have been advantageous as detection of *G. duodenalis* DNA may have been found by doing this. A methodology which included a concentration step of cysts from faeces before DNA extraction occurred within the samples may have increased detectable levels within this study. A method for this was developed by Wells *et al* (2016) for *Cryptosporidium* in adult bovine faeces; if this method worked for *G. duodenalis* then it would be worth investigating and including in future work.

Results of this study were also interesting as there were some animals which were very rarely found to be positive for *G. duodenalis* DNA. Animals 2403, 2411, 2415 and 2417 were only found to be positive for the presence of the parasite in single replicas and often only in single sample, despite a large number of samples being tested. Notably 2417, when detected, only had 1 of 2 replicates positive which could suggest that the levels of cysts in the faeces were lower compared to other animals. Animals 2411 and 2415 were also only detected as having a single replicate of a single sample positive for the parasite. These animals were found to only be positive within the samples taken at 6 months. This would suggest that the animals would most likely have became infected earlier in their life, however either sampling within this study missed days of shedding or the shedding levels were lower than detectable by this qPCR. The majority of animal samples, where tested, were positive for the parasite DNA on more than one occasion. In total 6 animals were found to be negative in each sample for the parasite DNA. Of

these animals, some had more samples tested than others and so to confirm this result in some animals additional samples would need to be investigated by the qPCR. Interestingly, animal 2402 had every sample available tested and it was found to be completely negative for parasite DNA detection. It is still unknown whether this animal has remained truly negative from birth until sampling end, as it could be again that shedding dates have been missed or was beyond detection threshold. It would be unlikely that these negative animals are truly negative, as infection has often been reported to be very high in farm environments (Chapter 1).

This study would benefit from samples which were collected soley for the purpose of completing the aim of this section of work; the identification of *G. duodenalis* within bovine faecal samples. As samples were used which were collected for a different study, there would have been limiations involved with the samples. A longer time frame of sampling would have been desirable, along with increased sampling freqeuency at later dates thoughout the work. Although the 6 month samples were only available on one date and did highlight some positive samples, these were not enough to reliably estimate the levels of infection within the cattle at this timepoint due to sporadic shedding of cysts in cattle. Inclusion of a concentration method for *G. duodenalis* cysts, as proposed previous (Wells *et al*, 2016) could be incorporated into the study. This would have increased the sensitivity of the sample testing as potentially increased levels of DNA would have been within samples. This could have increased all aspects of the molecular methods involved within the study, which could have allowed typing of positive samples found.

3.3.3 *Conclusion*

In conclusion, this study found that there was substantial *G. duodenalis* infection within dairy cattle of this farm however very little evidence of the parasite within water was found. This does not agree with previous work carried out within Scotland suggesting that there may be more to the story than this study has found. A detailed exploration of possible factors which could have had an effect to limit the levels of *G. duodenalis* DNA within water samples, other than those already discussed, would be useful. Furthermore, a repeat of the study using samples specifically collected and processed to analyse the levels of *G. duodenalis* within water samples around Scotland would be advantageous. The samples in this study did however enable some insight into the levels of cysts within water in Scotland, with the two samples which were found to be very

weakly positive. It may be that much more sensitive assays are required to do this. This study highlights the care, which must be taken, when attempting to analyse water samples for *G. duodenalis* cysts/DNA as various factors from filtration to processing may impact success rates of the work as these steps may impact *G. duodenalis* cyst integrity.

Although not every animal within the study was found to be infected, the majority of animals were positive at some point during the sampling period analysed in this study. Processing of additional samples would have most likely yielded increased numbers of animals that tested positive for the infections. Increased sampling and adaptations of processing of samples to increase the sensitivity of the test would have yielded more positive results at later stages in life - especially in animals which have previous infections in the past. Animals within this study were first noted to have samples positive for *G. duodenalis* DNA from as soon as 5 days after birth (animals 2384 and 2385). A paper by Mark-Carew *et al* (2010) has detected a similar situation, with infection detected as early as 2 days, showing that the situation is Scotland is probably no different in terms of initial infection of young animals. The work combined with the young age of infection would suggest that environmental contamination is vastly prevelant within farm environments as animals must be exposed to the parasite immediately from birth in some cases, if not shortly after. Adult cattle will most likely serve as a source of infection for their young, as prevelance of giardiasis within herds is known to be almost 100% (see Chapter 1). Optimisation of the qPCR to assess the specific assemblage of parasite within samples is crucially important when assessing the risk of the parasite to public health. Zoonotic transmission possibility as well as frequency for *G. duodenalis* is of much interest to public health bodies, therefore it would be advantageous to spend time to optimise the use of both the n-PCR and qPCR, which didn't work as expected within this study, and to re-analyse the samples. In doing so, identification of any assemblages with risk to human infection (A and B) could be then detected and an idea of their spread within both water catchments and cattle resevoirs could be assessed.

4. Chapter 4 – General Discussion

4.1 Discussion of Overall Results

This study investigated the presence of *G. duodenalis* within environemental samples as well as ways of improving current water filteration methodologies for the parasite, through the incorporation of megasonic sonication within a commonly used and approved method by the Drinking Water Inspectorate (DWI) within the UK.

Investigation into the use of megasonic sonication with the existing FiltaMax system found that the intergration of the two methods looked to be promising. Currently, as the megasonic method is in its infancy, the recoveries were not able to surpass the well-optimized and developed FiltaMax system. Further optimization and validation of the megasonic sonication for the elution of *G. duodenalis* would be benefitial as the recoveries achieved were very close to the standard method. Identifying areas of loss and adding steps to reduce their impact will no doubt be an important part of this process, as well as the design of a simpler method of exposing the filter modules containing parasite to the megasonic sonication. Despite not performing as well as the standard method, it was found that the megasonic method was significantly better when reducing the numbers of damaged cysts within sample. This could have large implications when the method is optimized as in doing so it will increase the numbers of reportable *G. duodenalis* cysts which water companies would observe. Cysts which have no contents will not be reported as a possible threat to the public by a water analyst, however it is unknown whether this means the cyst shell was empty before filtation or if the filtration process itself has caused damage to the cyst. This could lead to false positives which is very undesirable within a system build to protect public health. Another beneft of the megasonic combined method is that is it substantially easier for use. The standard FiltaMax method has many manual steps involved, which can be skipped entirely by the new megasonic method. This both assists in water laboratory sample processing efficiency, as well as opens doors to potential full automatic processing of filters in the future. Automation of water testing is something which is desirable, as it would both benefit a water authority in terms of efficiency and cost of sample processing.

Further work must still be done to move the megasonic sonication method to a stage when automation can be envisaged, notably with field trials in which not only live cysts are used; but cysts that may be environmentally strained. This area could be where

the megasonic method shines through, due to its gentler elution method which could avoid further damage to already damaged, but still infective, cysts. This is something which was speculated to be an issue in creating a combined *Cryptosporidium* / *Giardia* filtration method in the past, which could be overcome by the megasonic method (Chapter 2).

Damage of cysts was something which could have also contributed to the low levels of cyst DNA found within Chapter 3 of this work. The number of stages in which the water samples were subjected to before reaching The Moredun Research Institute could have had an impact on cyst integrity, causing DNA to be lost throughout the wash steps before the DNA extraction process. This will be similar to when sampling water to be investigated for *G. duodenalis* in a real outbreak of the parasite, which could affect the reliability of outbreak investigations.

It has been highlighted by this study, in combination with many others studies throughout the world, that *G. duodenalis* infection is widespread throughout cattle populations. Further investigations into the presence of specific assemblages and sub-assemblages is paramount in assessing public health risk, however this study was unfortunately unable to determine that risk. A global effort must be made to overcome the current contradictory data which only creates confusion with regards to the true epidemiology and threat of *G. duodenalis* to the public. Standardisation of techniques used by studies, identification of specific assemblages causing infection (and sub-assemblages) within studies and forecasting how the information affects public health within the area are crucial for the future. It is also especially important that these aspects of epidemiology are understood as from literature if appears that often geography can have a large impact on many factors of the parasite. Host cycle, dominant assemblage and levels of transmission all appear to vary dependant on the location in the world. Overcoming these differences and understanding how these factors occur or are connected will be most useful when unravelling the epidemiology of the parasite.

Global collaborations into education, notably for farmers will be beneficial in order to limit widespread contamination of agricultural land by cattle, which can often reach water catchments easily due to lack of restricted grazing near to watercourses. Notably, this is crucial within Scotland and indeed the UK and other countries that rely heavily on surface water for their drinking water supply. Increased rainfall will wash cysts more effectively from pasture into steams and rivers leading to spread of the parasite and contamination of catchments and areas downstream, which in turn threatens

a wide range of domestic, livestock and wild animals (assemblage A of the parasite) which then in turn threatens public health.

5. Appendices

5.1 Appendix A: Scottish Water Sample Listings.

Samples included in this appendix are only those in which testing was carried out.

Legend

***R = Raw Water; F = Finished Water**

: Negative Sample

: Positive Sample by β-Giardin qPCR

: Not enough sample or sample missing

Sample number	SW sample ID	Water Type*
1027	Bonnycr G-161013	F
1028	Coulter G-151013	F
1029	Bmorhi G-161013	F
1031	Rawburn G-221013	F
1033	Perth_G G-221013	F
1036	Howden G-221013	F
1038	Smoorh G-171013	F
1358	Mannofi G-241013	F
1359	Balmore G-301013	F
1360	Flasov G-301013	F
1361	Glenlat G-231013	F
1362	Mannofi G-301013	F
1363	Daer2 G-011113	F
1364	Lumsden G-301013	F
1364	Craighe G-241013	F
1365	Kenmore G-041113	F
1366	Roseber G-231013	F
1367	Pen G-041113	F
1368	Kilchoan G-271013	F
1369	Balmore G-291013	F
1370	Bcrks G-051113	F
1371	Lomond G-291013	F
1372	Glendev G-301013	F
1373	Douglas G-051013	F
1374	Bradan G-291013	F
1375	Knowehe G-051113	F

1376	Cnure G-231013	F
1377	Bmichl G-051113	F
1378	Newcast G-051113	F
1379	Milngavi G-231013	F
1380	Clatto G-051113	F
1381	Craighe G-301013	F
1382	Glenfar G-291013	F
1384	Tomnavo G-141113 ERA	F
1385	Tomnavo G-141113 RKS	F
1387	Tomnavo G-061113 ERA	F
1388	Tomnavo G-061113 RKS	F
1390	Tomnavo G-101113 ERA	F
1391	Tomnavo G-101113 RKS	F
1393	Tomnavo G-131113 ERA	F
1394	Tomnavo G-131113 RKS	F
1397	Tomnavo G-181113 ERA007	F
1398	Tomnavo G-181113 ERA015	F
1400	Tomnavo G-121113 ERA	F
1401	Tomnavo G-121113 RKS	F
1403	Tomnavo G-081113 ERA	F
1404	Tomnavo G-081113 RKS	F
1406	Tomnavo G-151113 ERA	F
1408	Tomnavo G-171113 RKS	F
1410	Tomnavo G-211113 RKS	F
1412	Tomnavo G-191113 RKS	F
1414	Tomnavo G-071113 RKS	F
1415	Tomnavo G-071113 ERA	F
1417	Leck G-071113	F
1418	Herricks G-061113	F
1419	Herricks G-171113	F
1421	Leck G-141113	F
1423	Braemar G-121113	F
1424	Win G-211113	F
1425	Tomnavo G-111113 ERA013	F
1426	Tomnavo G-111113 ERA004	F
994	Private 6 G-161013 030	F
995	Private G-161013 031	F
996	Diavaig F G-151013	F
352	Leck G-05/09/13	F
972	Broadf G-161013	R
970	Teangue G-161013	R
997	Daer 2 G-101013 011	R
998	Daer 1 G-101013 015	R
1030	Rawburn C-221013	R
1032	Perth_G C-221013	R
1034	Coulter C-221013	R

1035	Howden G-221013	R
1037	Smoorh C-171013	R
1040	Kil C-171013	R
1042	Leck G-221013	R
1044	Glencor G-221013	R
1207	Pateshill C-311013	R
1209	Mannofi C-011113	R
1211	Lomond C-011113	R
1213	Castle_M C-291013	R
1215	Balmore G-291013	R
1217	Flasov C-301013	R
1219	Blinns G-301013	R
1220	Blinns C-311013	R
1221	Bradan C-031113 003	R
1222	Bradan C-031113 004	R
1224	Craighe C-231013	R
1226	Tullic C-231013	R
1228	Forehill C-231013	R
1230	Turriff C-301013	R
1232	Bcrks C-291013	R
1234	Tullic C-311013	R
1236	Leck G-311013	R
1238	Glencor G-311013	R
1240	Clatto C-291013	R
1242	Win C-251013	R
1244	Ascog C-311013	R
1246	Cnure G-301013	R
1248	Tomna C-281013 RKS	R
1250	Tomna C-041113 RKS	R
1255	Tomna C-241013 RKS	R
1256	Tomna C-031113 RKS	R
1259	Tomna C-011113 RKS	R
1262	Tomna C-291013 RKS	R
1264	Tomna C-281013 RKS	R
1269	Tomna G-231013 RKS047	R
1270	Tomna G-231013 RKS006	R
1272	Tomna G-271013 RKS	R
1273	Tomna C-271013 RKS	R
1274	Tomna C-031113 RKS	R
1275	Tomna G-031113 RKS003	R
1279	Tomna G-241013 RKS	R
1281	Tomna G-271013 RKS	R
1284	Westray C-231013	R
1285	Pen C-251013	R
1286	North H G-311013	R
1287	LVR C-011113	R

1288	Lintrath C-291013	R
1289	Broadfo C-311013	R
1290	Lintrath C-251013	R
1291	Invercam C-301013	R
1292	Tomnavo C-271013	R
1293	Kirkbiste C-011113	R
1294	Howdenh G-291013	R
1295	Chill C-231013	R
1296	Dhuloc C-311013	R
1297	Calder C-231013	R
1298	Balmore G-051113	R
1299	Boardho C-011113	R
1300	Calder C-311013	R
1301	Assynt C-241013	R
1302	Carronv C-041113	R
1383	Tomnavo C-141113 RKS	R
1386	Tomnavo C-061113 RKS	R
1389	Tomnavo C-101113 RKS	R
1392	Tomnavo C-131113 RKS	R
1395	Tomnavo C-181113 RKS001	R
1396	Tomnavo C-181113 RKS002	R
1399	Tomnavo C-121113 RKS	R
1402	Tomnavo C-081113 RKS	R
1405	Tomnavo C-151113 RKS	R
1407	Tomnavo C-171113 RKS	R
1409	Tomnavo C-201113 RKS	R
1411	Tomnavo C-191113 RKS	R
1413	Tomnavo C-071113 RKS	R

5.2 Appendix B: Cattle Sample Results & Relation to Cattle Age (weeks 0-9)

Positive Sample = , Negative Sample = , Sample Not Available = , Sample Not Tested =

Animal Number	2384		2385		2386		2387		2388	
Date	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
13-Jan										
16 January	0	0	0	0						
18 January	0	0							0	0
20 January	0	0								
23 January	0	0	0	0					0	0
25 January										
27 January	0	0								
30 January	0	1								
01 February										
03 February	0	0	0	0	0	0	0	0	0	0
06 February	0	0	1	1	1	1	0	0	1	0
08 February					0	0	0	0	0	0
10 February										
13 February			0	0	1	1	0	0	1	1
15 February	0	0	0	0					1	1
17 February										
20 February	1	1	0	0	0	0			0	0
22 February	0	0	1	1					0	0
24 February	1	1	0	0					0	0
27 February										
29 February										
03 March										
05 March										
07 March										
09 March										
29 March			0	0	0	0	0	0	0	0
02 May										
16 Jul			0	0					0	0

Animal Number	2389		2390		2393		2394		2395	
Date	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
13-Jan										
16 January										
18 January										
20 January										
23 January										
25 January										
27 January										
30 January										
01 February					0	0				
03 February			0	0					0	0
06 February	1	0	1	1	1	1			1	0
08 February	1	1	0	0	1	0	1	1	0	0
10 February										
13 February			0	1						
15 February	0	0			1	0				
17 February										
20 February										
22 February			0	0	0	0			0	0
24 February			0	0						
27 February										
29 February										
03 March										
05 March										
07 March										
09 March										
29 March	0	0	0	0						
02 May										
16 Jul					0	0				

Animal Number	2396		2397		2398		2399		2402	
Date	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
13-Jan										
16 January										
18 January										
20 January										
23 January			0	0						
25 January					0	0	0	0	0	0
27 January							0	0	0	0
30 January									0	0
01 February							0	0	0	0
03 February			0	0			0	0	0	0
06 February									0	0
08 February							0	0	0	0
10 February										
13 February							0	0		
15 February							1	1	0	0
17 February										
20 February	0	0	0	0			1	0	0	0
22 February	0	0					0	0	0	0
24 February			0	0			0	0	0	0
27 February							0	0	0	0
29 February							0	0	0	0
03 March							0	0	0	0
05 March										
07 March										
09 March										
29 March										
02 May			0	0					0	0
16 Jul			0	0					0	0

Animal Number	2403		2404		2405		2407		2409	
Date	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
13-Jan										
16 January										
18 January										
20 January										
23 January										
25 January	0	0								
27 January	0	0								
30 January	0	0								
01 February	0	0	0	0	0	0				
03 February	0	0					0	0		
06 February	0	0								
08 February	0	0			0	0				
10 February										
13 February										
15 February	1	0								
17 February										
20 February							0	0		
22 February					0	0				
24 February	0	0			0	0				
27 February	0	0	0	0	0	0	0	0		
29 February			0	0	0	0	0	0		
03 March	0	0	0	0	1	1	0	0		
05 March	0	0	1	1	0	0	0	0		
07 March			0	0	0	0			0	0
09 March					1	1				
29 March										
02 May										
16 Jul	1	1	1	1			0	0		

Animal Number	2411		2412		2413		2415		2417	
Date	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
13-Jan										
16 January										
18 January										
20 January										
23 January										
25 January										
27 January										
30 January										
01 February							0	0		
03 February	0	0					0	0	0	0
06 February							0	0		
08 February	0	0					0	0	0	0
10 February										
13 February							0	0	0	1
15 February	0	0					0	0	0	1
17 February	0	0							0	0
20 February	0	0					0	0	0	0
22 February	0	0					0	0		
24 February	0	0					0	0	0	0
27 February					0	0	0	0	0	0
29 February	0	0					0	0		
03 March	0	0					0	0	0	0
05 March	0	0			0	0	0	0	0	0
07 March									0	0
09 March							0	0	0	0
29 March										
02 May	0	0					0	0		
16 Jul	1	0					1	0	1	0

Positive Sample = , Negative Sample =

	13/01/2012	16/01/2012	18/01/2012	20/01/2012	23/01/2012	25/01/2012	27/01/2012	30/01/2012	01/02/2012	03/02/2012	06/02/2012	08/02/2012	10/02/2012
Calf ID													
2384	2	5	7	9	12	14	16	19	21	23	26	28	30
2385	2	5	7	9	12	14	16	19	21	23	26	28	30
2386	1	4	6	8	11	13	15	18	20	22	25	27	29
2387	1	4	6	8	11	13	15	18	20	22	25	27	29
2388	1	4	6	8	11	13	15	18	20	22	25	27	29
2389		2	4	6	9	11	13	16	18	20	23	25	27
2390		1	3	5	8	10	12	15	17	19	22	24	26
2393			2	4	7	9	11	14	16	18	21	23	25
2394			1	3	6	8	10	13	15	17	20	22	24
2395			1	3	6	8	10	13	15	17	20	22	24
2396			1	3	6	8	10	13	15	17	20	22	24
2397				2	5	7	9	12	14	16	19	21	23
2398				2	5	7	9	12	14	16	19	21	23
2399				2	5	7	9	12	14	16	19	21	23
2402						2	4	7	9	11	14	16	18
2403						2	4	7	9	11	14	16	18
2404						1	3	6	8	10	13	15	17
2405						1	3	6	8	10	13	15	17
2407							2	5	7	9	12	14	16
2409								2	4	6	9	11	13
2411								1	3	5	8	10	12
2412									2	4	7	9	11
2413									2	4	7	9	11
2415									2	4	7	9	11
2417									1	3	6	8	10

13/02/2012	15/02/2012	17/02/2012	20/02/2012	22/02/2012	24/02/2012	27/02/2012	29/02/2012	03/03/2012	05/03/2012	07/03/2012	09/03/2012
33	35	37	40	42	44	47	49	52	54	56	58
33	35	37	40	42	44	47	49	52	54	56	58
32	34	36	39	41	43	46	48	51	53	55	57
32	34	36	39	41	43	46	48	51	53	55	57
32	34	36	39	41	43	46	48	51	53	55	57
30	32	34	37	39	41	44	46	49	51	53	55
29	31	33	36	38	40	43	45	48	50	52	54
28	30	32	35	37	39	42	44	47	49	51	53
27	29	31	34	36	38	41	43	46	48	50	52
27	29	31	34	36	38	41	43	46	48	50	52
27	29	31	34	36	38	41	43	46	48	50	52
26	28	30	33	35	37	40	42	45	47	49	51
26	28	30	33	35	37	40	42	45	47	49	51
26	28	30	33	35	37	40	42	45	47	49	51
21	23	25	28	30	32	35	37	40	42	44	46
21	23	25	28	30	32	35	37	40	42	44	46
20	22	24	27	29	31	34	36	39	41	43	45
20	22	24	27	29	31	34	36	39	41	43	45
19	21	23	26	28	30	33	35	38	40	42	44
16	18	20	23	25	27	30	32	35	37	39	41
15	17	19	22	24	26	29	31	34	36	38	40
14	16	18	21	23	25	28	30	33	35	37	39
14	16	18	21	23	25	28	30	33	35	37	39
14	16	18	21	23	25	28	30	33	35	37	39
13	15	17	20	22	24	27	29	32	34	36	38

5.3 Appendix C: Paper will be submitted to the Journal: Water Research

Towards Automated Systems for Waterborne Protozoa Elution with Megasonic Enhancement

Horton, B.[1,2,3], Kerrouche, A.[2,4], Katzer, F.[1], Desmulliez, M.[4] and Bridle, H.[2]

1. Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ

2. Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS

3. Moredun Scientific, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ

4. Institute of Sensors, Signals and Systems, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS

Keywords: waterborne protozoa, automation, filtration systems, megasonic elution, monitoring

Abstract: Continuous and reliable monitoring of water sources for human consumption is imperative for public health. For protozoa, which cannot be multiplied efficiently in the lab, concentration and recovery steps are key to a successful detection procedure. Recently, megasonic waves have been employed to efficiently recover *Cryptosporidium* from commonly used water industry filtration procedures, forming a basis for a simplified and more effective method of elution of pathogens. Sonication methods are already employed in many scientific fields of science as a method of cleaning equipment, disrupting cells or killing pathogens, to name only a few uses. In this paper, we report the benefits of incorporating megasonic sonication into the current methodologies of *Giardia duodenalis* elution from an internationally approved protocol used within the water industry, the FiltaMax system. The megasonic protocol has many benefits over the current method, including but not limited to; a reduced final volume of eluent allows removal of time-consuming centrifugation steps and a reduction in manual involvement resulting in a more consistent and potentially cheaper method. We also show that megasonic sonication of *G. duodenalis* cysts provides the option of a less damaging elution method compared to the standard FiltaMax system, although currently the elution from filter matrices is not fully optimised. A notable decrease in recovery of damaged cysts was observed in megasonic processed samples, potentially increasing the abilities of further genetic identification options upon isolation of the parasite from a filter sample. Additionally, we explore the potential of megasonic elution with other filter types with *Cryptosporidium* demonstrating increases in recovery rate for easily automatable set-ups. This work paves the way for development of a fully automated method for protozoa elution from water samples.

Introduction

Giardia duodenalis is a waterborne pathogen which causes both an economic and a public health burden throughout the world and is thought to infect upwards of 200,000,000 people per year (Lane & Lloyd, 2002). It is a common diarrhoeal causing protozoan parasite, particularly in developing countries and causes the disease called 'giardiasis' (Julio *et al*, 2012). The transmission route of the parasite is faecal/oral and parasite cysts are found within surface waters throughout the world. Although the disease is much more prevalent in the developing world, outbreaks of disease do still occur in the developed world, albeit at a lower level (Feng & Xiao, 2011; Barwick *et al*, 2000). A study of waterborne disease outbreaks between 2004-2010 reported that *G.duodenalis* accounted for 70 of 199 reported outbreaks of human disease due to waterborne protozoa within the developed world (Baldursson & Karanis, 2011). In addition, food can easily become contaminated when washed with water containing infective cysts, emphasizing implications for food hygiene worldwide (Porter *et al*, 1990; Smith *et al,* 2007).

Cysts are well recognized as being environmentally resistant as well as resistant to commonly used water treatment methods (Robertson & Lim, 2011b). Quantities of cysts required to cause infection vary dependant on host but in humans typically the infectious dose is between 10-100 cysts, subject to the immune status of individual hosts (Roxstrom-Lindquist *et al*, 2006). Data collaborated by Robertson & Lim (2011a) suggests humans and cattle infected with *G. duodenalis* can shed upto $1x10^6$ and $1x10^5$/ gram of faeces, respectively. The quantities of immediately infectious cysts shed by hosts, combined with impressive cyst longevity (can remain infective from weeks to months in the environment) highlight concerns for public health and widespread environmental contamination.

Currently there are regulatory requirements for water within the UK for a similar protozoan species, *Cryptosporidium*, which has been extensively monitored in water since their introduction under the Water Supply (Water Quality) Regulations 1999, SI No. 1524. The UK regulations, enforcing the use of specific filtration methods, have had a positive impact on reducing outbreaks of *Cryptosporidium* in the public and data presented by Robertson & Lim (2011c), on work by Lake *et al* (2007), suggests that in North England 905 cases (~7000 infections) were prevented since its implementation to 2007. Although not monitored within the UK, *Giardia duodenalis*, amongst a large number of other water contaminants, is extensively monitored within the USA. Following the EPA Safe Drinking Water Act (SDWA) in 1997 *Cryptosporidium* and *Giardia* became extensively monitored and regulated using US EPA Method 1623 (Method 1623.1, 2012). These introductions have been very effective in reducing outbreaks within the USA where there have been more reported outbreaks of giardiasis than anywhere else worldwide (Robertson & Lim, 2011d). Delay between point of infection and clinical disease, and varying clinical symptoms displayed in

individual infections lead to potential large-scale under-reporting of the parasite throughout the UK, and indeed the world.

Filtration of *G. duodenalis* from water samples is currently carried out using the same methodology as that for *Cryptosporidium* in the UK - the FiltaMax system. Current filtration recoveries for *Giardia duodenalis* cysts in reagent grade water is said to be between 41-70% referring to the UK Environment Agency (2010), something which has been confirmed by many additional authors over the years with mean recoveries of 49.8 +/-5.4%, 56.7+/-22.2%, (Wohlsen *et al* (2004), http://www.dwi.gov.uk/research/reports/DWI70-2-155_giardia.pdf;). It has also been speculated that *G. duodenalis* cysts, being not as resistant as *Cryptosporidium*, may be damaged or destroyed by the process of filtration and/or elution using the Filta-Max System. This was noted as a possible source of failure to create a dual-monitoring method for both *Cryptosporidium* and *Giardia* in 2002, which was investigated by Severn Trent Labs on behalf of the Drinking Water Inspectorate (DWI) in the UK (http://www.dwi.gov.uk/research/reports/DWI70-2-155_giardia.pdf). This report highlights the need for the further development of a filtration process which can reliably recover *G. duodenalis* cysts from samples and also be combined with other species effectively. A method incorporating as megasonic sonication has the potential to for fill this niche.

Megasonic sonication operates in a similar way to that of ultrasonic sonication, however a much higher frequency of sound waves are used. Typically megasonic sonication involves a frequency of over 1 MHz created by a piezoelectric transducer which is placed within a fluid filled container. When powered the transducer creates high frequency sound waves which move through the fluid and oscillate through maximum and minimum pressures at points along the wave. At the point where the minimum pressure along the wave is below the vapour pressure of the liquid bubbles form; these bubbles then collapse upon exposure to the maximum pressure of the wave. This formation and implosion of bubbles creates a much kinder elution process compared to that of ultrasonic sonication, due to the bubbles being smaller in size and thus creating less local turbulence when collapsing (Kerrouche *et al,* 2015; Chitra *et al*, 2004). Megasonic sonication has shown promising results in elution of *Cryptosporidium parvum* from Filta-Max matrixes, as reported by Kerrouche *et al* (2015). The method allowed recoveries of the common waterborne pathogen comparable to that of the Filta-Max methodology (commonly used within water testing internationally, including the UK (The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14). The megasonic technique however displayed a range of benefits, e.g. time and resource saving, removal of non-automatable steps, over the current method.

In an attempt to improve the current methods of *G. duodenalis* filtration using the FiltaMax system, here we have incorporated megasonic sonication into suitable steps of The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14

standard protocol of *Giardia duodenalis*, and investigated if the same benefits seen when eluting *Cryptosporidium* by the authors in *Kerrouche et al (*2015) are evident when used to elute gamma-ray inactivated *G. duodenalis* cysts from the FiltaMax system. In addition, we examined the impact of the megasonic sonication method with regard to damages and effects on inactivated *G. duodenalis* cyst stability during elution.

One key outcome of the previous *Cryptosporidium* study was the potential for a more automatable approach using megasonic elution. Currently an automatic version of the Filta-Max wash station is available which is commonly utilized by water companies in an attempt to both reduce the level of manual activity required by analysts processing samples, as well as standardizing the elution process by removing human involvement. This system however cannot be used for the latter stages of the method, meaning variation through human error still exists along with the level of manual activity required. Other methodologies for filtration of parasites from water sources (Wohlsen *et al*, 2004; McCuin & Clancy, 2003) have been explored with a range of different filter types. Here, we also investigated the potential of megasonic elution with alternative filter types in two different automated filter set-ups to demonstrate the wider applicability of this method and confirm the automatable advantages.

Materials and Methods

Three areas of potential were investigated for the inclusion of megasonic sonication with *Giardia* (Figure 2 for a schematic of the megasonic bath set-up) for improvement of the original method as found in The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14 (see Figure 1 for an overview of the method and megasonic steps investigated). Following these a complete method for using megasonic sonication was developed using ideas both from Kerrouche *et al* (2015) and experience from shortfalls from the initial three areas of potential for megasonic sonication: direct seeding of *Giardia duodenalis* cysts onto the FiltaMax membrane, seeding *Giardia duodenalis* into PBST eluent before concentration through a FiltaMax membrane and finally seeding *Giardia duodenalis* into sponges within a FiltaMax module.

In all experiments samples were spiked where appropriate with EasySeed suspensions containing 100 gamma-irradiated *Giardia duodenalis* cysts, purchased from TCS Biosciences, UK. The megasonic transducer used for this work was purchased from Sonosys (Sonosys, 2016) and FiltaMax modules and membranes were purchased from IDEXX (IDEXX Company, 2016). Each sonication step where required was carried out for 20 minutes as standard, an exposure time taken from Kerrouche *et al (2015).* The resulting slides from each experiment were then stained using a monoclonal antibody as per manufacturer's instructions (Giardia-Cel Reagent – TSC Biosciences, excitation 490nm; emission 510nm) and a nucleic stain (DAPI (4',6-diamidino-2-phenylindole) – Fisher, UV excitation 355nm, emission 450 nm) at a concentration of 2mg/mL diluted to 1:5000 in Phosphate Buffered Saline (PBS). Following staining slides were mounted

and sealed with nail varnish before being then counted using both fluorescence and DIC (Differential Interference Contrast) microscopy, on an Olympus BX50 with BX-FLA and DIC attachments. The total numbers of cysts were then recorded.

Figure 1— Flow Diagram of Differences of Standard and Megasonic Methodologies

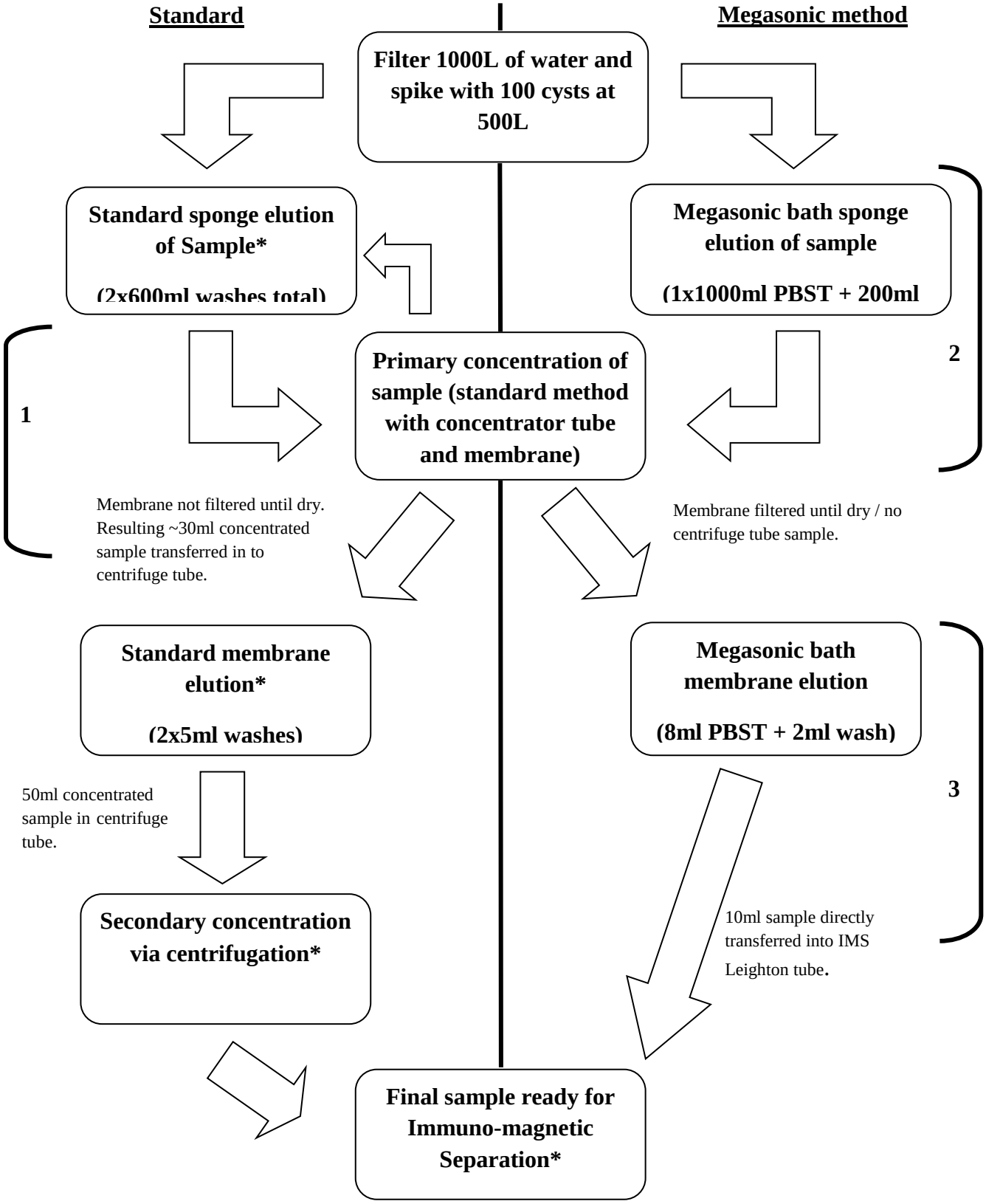


Figure 1— This flow diagram shows the differences in the key stages in the sample elution using the standard Filta-Max methodology and the novel Megasonic method. **Bracket 1** indicates the steps investigated by seeding directly into the 600 PBST and processing using standard and megasonic techniques. **Bracket 2** indicates the steps investigated by carrying out sponge seeding experiments using standard and megasonic techniques. **Bracket 3** indicates the steps investigated by carrying out direct seeding onto membranes, allowing a difference to be investigated between standard and megasonic techniques when removing potentially embedded cysts.

**** Carried out following** The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14*

Figure 2—Diagram of the Megasonic Bath Setup for *Giardia*

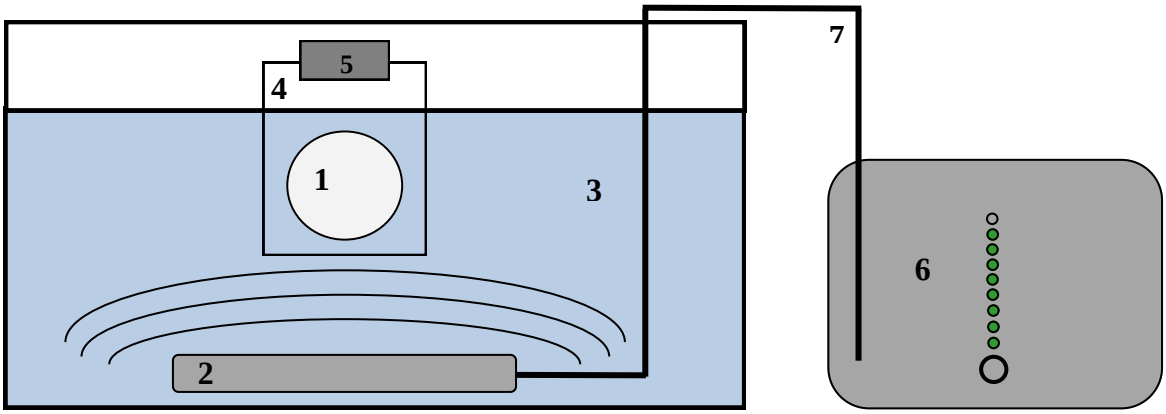


Figure 2— A diagram showing the setup of the megasonic bath during membrane sonication. Samples can be seen submerged in the water bath and exposed to megasonic waves generated by the megasonic transducer. Samples were sonicated for 20 minutes in this fashion. 1) FiltaMax membrane 2) Megasonic transducer generating megasonic waves 3) Water filled bath 4) Partially submerged plastic bag containing PBST and membrane 5) adhesive tape between the water bath and plastic bag 6) Megasonic control and power supply 7) Water protected cabling.

Cysts were also exposed to megasonic waves prior to their viability being assessed using propidium iodide (PI). This allowed insight into if megasonic waves had any effect on the viability of live *Giardia duodenalis* cysts.

The megasonic exposure effects were investigated on two time points – Day 1 of exposure and day 7 following exposure. This meant any delayed effect that the megasonic exposure may have, for up-to a week, could be investigated. A non-exposed control sample was also included with these tests on both days which were not exposed to megasonic waves. Samples were stained using Propidium Iodide (Fisher, UV 535-nm excitation, >590-nm emission), DAPI (4',6-diamidino-2-phenylindole) and a monoclonal antibody (FITC-CmAb) (Giardia-cel Reagent) and then mounted on a clean microscope slide. The slides were viewed using an Olympus BX50 with BX-FLA with a DIC attachment and 100 cysts total were counted in each sample and a note made of their status (viable/non-viable). Propidium iodide will only pass into the internal contents of the cyst if the parasite has lost the ability to control its cyst membrane, which occurs when a cyst is dead. The cysts were therefore deemed to be dead if they had been stained with Propidium Iodide and live if they had not been stained. The FITC CmAb and DAPI stains helped with location of the cysts themselves and the nuclei of the trophozoites inside them, respectively. Empty cysts were not counted within the test.

Finally, megasonic use with different filter types was investigated using *Cryptosporidium* (Moredun isolate). Details of all the different experimental conditions and set-ups are described below. In each case the standard protocol was compared to megasonic use, at some point in the protocol, and three replicates were always performed for each set of conditions.

Giardia duodenalis Seeding into Sponge Filter Matrices

Filters were prepared for seeding as standard (UK Environment Agency (2010) Microbiology of Drinking Water: Part 14) and 1000L of water was passed through them. Half way through the filtration (500L) a spike of 100 *Giardia duodenalis* cysts was injected into the filter. The 10mL spike was created by drawing the cyst suspension (made up to 5mL), plus a 2mL wash, into a 10mL syringe and adding ultrapure water to make the spike up to 10mL total volume. Syringes were numbered and filter housings unique identifiers were documented to allow traceability following spiking.

Following this filters were either processed using a megasonic method or the standard method, as can be found in The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14. The megasonic method involved removing the filter module from the filter housing and removing the screw holding the filter membrane together. The filter sponges, now loose, should be placed into a large plastic bag containing 1200 mL PBST. The sponges should be separated from each other when inside the bag when possible to promote removal of cysts from the matrices. The plastic bag, now containing the separated sponge filters, was placed into the megasonic bath and sonicated for 20 minutes set to 2MHz. Following this the bag was removed from the megasonic bath and the liquid poured into a concentrator tube containing a magnetic stirrer. This was then concentrated down until a small amount of liquid remained above the membrane. The bag was then cut at one corner and squeezed to remove any remaining PBST containing cysts into the concentrator tube. The liquid was again concentrated down to a small level above the membrane. The liquid was then poured into a centrifuge tube for later use, along with a small amount of PBST to wash the concentrator tube. From this point the membrane was processed as usual following The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14.

Giardia duodenalis Seeding into 1200mL PBST for Membrane Processing

The direct seeding experiments investigated the abilities of megasonic sonication to remove *G. duodenalis* cysts from membranes upon direct seeding. This section of work allowed insight into how the volume of eluent containing the cysts would affect the abilities of the megasonic to recover cysts from the membranes. Six samples were prepared in the following manner. A FiltaMax concentrator tube base was fitted with a FiltaMax membrane and the concentrator tube was screwed in as would be done as standard. To this tube 600mL PBST was added in accordance with the standard protocol and a spike of 100 *Giardia duodenalis* cysts was added (as above made up to 5mL and followed by a 2mL wash). The sample was then concentrated through the membrane as standard using a magnetic stirrer. Following a small amount of liquid remaining above the membrane, an additional 600mL PBST was added to the tube (to represent the second wash as per the standard protocol) and concentrated down to

the same low level (now totalling 1200mL PBST used). The magnetic stirring bar was removed and washed using a small amount of PBST to remove any cysts that may have been attached. The sample was again concentrated briefly until a low level of eluent remained above the membrane surface. From this point three samples were processed as per the standard methodology and three were processed using megasonic sonication.

Following the standard method, the sample was decanted from the concentrator tube into a centrifuge tube and the membrane was added into a bag with 2x5mL PBST washes, before the washes being added into the same centrifuge tube as the concentrator tube sample eluent. These were then made up to 50mL and centrifuged and prepared for IMS processing following the standard method.

The megasonic method involved direct transfer of the remaining concentrator tube eluent into the plastic bag which would contain the membrane for processing. The concentrator tube was then washed briefly with 10mL PBST and again decanted into the plastic bag. The membrane was then removed from the concentrator tube and carefully placed into the same plastic bag (now containing ~50mL eluent). This was then sonicated in the same method as the previous experiment, but with 50mL PBST as opposed to 10mL as previous (see figure 1.). Following the megasonic exposure the eluent from the bag was transferred into a centrifuge tube and centrifuged and prepared for IMS processing as standard.

***Giardia duodenalis* Direct Membrane Seeding**

This section of the work allowed an understanding of whether the megasonic elution would satisfactorily recover cysts potentially embedded within the FiltaMax membrane which would be utilized upon concentration of the eluent used to wash the FiltaMax module. The base of the FiltaMax concentrator tube (without the tube) was fitted with a FiltaMax membrane and a hand pump before being primed using PBST (Phosphate Buffered Saline / Tween 20). The 100 cyst spike was then made up to 5mL using PBST, passed through the membrane using an appropriate pipette whilst applying pressure using the hand pump.

A wash of 2mL PBST was then added to the tube and thoroughly mixed before being passed through the same membrane. Care was taken when passing the spike through the filter to ensure that pressure did not exceed levels comparable to the standard method.

Six sample membranes were prepared in this manner and from these three were processed using the standard rubbing method using 2x5mL PBST washes in a plastic bag (as supplied with IDEXX FiltaMax membranes) as controls with the eluent from each wash transferred into a single labelled centrifuge tube. Following this the total 10mL from the control filter membranes were each made up to 50mL using ultrapure

water and centrifuged as standard, prior to following the protocol onto the IMS stage of processing. The final three sample membranes were processed using megasonic sonication inside of a water bath, instead of the standard method of rubbing. These sample membranes were placed in a standard plastic bag, as above, containing 10mL PBST and taped to the side of the megasonic bath, ensuring the total eluent wash was immersed within the water (see Figure 1.). Samples were sonicated for 20 minutes, which was based on the paper by Kerrouche *et al* (2015) as an optimum exposure time. The 10mL sample was then transferred directly from the plastic bag into a Leighton tube and from this point IMS was carried out following the standard protocol, using an Invitrogen Dynabeads® G-C Combo kit. An additional sample was also processed as a control (non-megasonic method) on centrifugation, to assess if this had any effect on the cysts recovered via this method. In this work a single sample was processed following the standard protocol, however the 2x5mL washes were placed directly into a Leighton IMS tube and processed using IMS.

A Complete Procedure of *Giardia duodenalis* Elution using Megasonic Sonication

The filters containing known quantities of *Giardia duodenalis* cysts were prepared in the same manner as the section above, using suspensions containing 100 gamma-irradiated cysts. Six filters were prepared in total, with three acting as controls to be processed according to the standard methodology found in The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14. The final three were processed using a methodology developed to incorporate megasonic sonication fully into the elution process. Adaptations were made to the procedure following results obtained from previous work, which included wash steps being incorporated into the existing method. When sonicating filter sponges (again in the same method as previous section) a PBST volume of 1000mL was utilized, instead of 1200mL. Following sonication, the PBST was poured from the bag into the concentrator tube (containing a filter membrane) and the sponge disks were wrung out and then concentrated down to a lower volume using the hand pump. A final wash of 200mL PBST was then added to the bag to recover any cysts still remaining in the plastic bag or sponge disks. The wash 200mL PBST was then manoeuvred briefly inside the bag, before being poured into the same concentrator tube as the 1000mL and the disks wrung out again.

The concentrator tube containing the membrane and eluent was then drained down slowly to a point in which the membrane was dry, being careful not to damage the membrane itself. The membrane was then removed and added into a plastic bag containing 8mL PBST, which was then placed into the megasonic bath to be sonicated for 20 minutes. This 8mL eluent was then transferred directly into an IMS Leighton tube and a further 2mL wash of PBST was added to the bag to recover any cysts which had been left behind in the first wash. This 2mL wash was then removed from the bag

and transferred into the same IMS Leighton tube, to be processed as standard following The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14.

Megasonic Elution in Automated Set-Ups with Different Filter Types

Two different filter types were trialled. The first was a Rexeed XA25 (Asahi Kasei) used in a dead-end filtration set-up. 10L was spiked with 100 *Cryptosporidium* oocysts (Moredun isolate, counted and supplied by Scottish Water) and the backflush solution utilised was 500mL of PBST. The collected backflush was analysed at Scottish Water using the standard method starting from the membrane concentration stage, following The UK Environment Agency (2010) Microbiology of Drinking Water: Part 14. The second filter was a Fresenius FX1000 in a tangential flow system. Again 10L was spiked and used as the starting solution. In this case the sample was recirculated until 100mL was remaining. The concentrate was also analysed at Scottish Water with the standard method as above. Megasonic conditions were as described above.

Results and Discussion

***Giardia duodenalis* Seeding into Sponge Filter Matrices**

The investigation into the efficiency of removing *G. duodenalis* cysts from filter sponges using megasonic sonication yielded acceptable results showing only a minor difference between the performance of the two methods in terms of recovery rate (Table 1 and Figure 3A). When taking into account all counted objects the recovery rates were 23% and 16%, for the standard and megasonic methods, respectively. It was also observed that the filter sponges in both megasonic and control sponge experiments expanded poorly, which could account for the slightly lower recovery of cysts during this experiment compared to the estimated 41-70% expected recovery as noted by UK Environment Agency, page 82 (2010).

However, it was notable that the standard method resulted in several shells being observed whereas this was not the case for the megasonic method. Standard procedure within a water laboratory is that a cyst shell is regarded as a non-reportable object and thus not counted as an organism. These are often reported as '*giardia* like-bodies' or 'glbs' in a comment or side note for the sample, but not included in a total count of cysts within the sample as they do not pose a risk to public health. Due to this the integrity of cysts recovered from a water samples is of crucial importance to assessing threat to public health within a sample from a catchment. Considering the number of intact cysts counted after each protocol the recovery rates were 20% and 16%, for the standard and megasonic methods, respectively. The difference here was not found to be statistically significant ($p=0.146$ using a One-Way ANOVA). Therefore,

it is evident that the use of megasonic elution as an alternative to the standard procedure offers a similar recovery rate for this step of the protocol.

***Giardia duodenalis* Seeding into 1200mL PBST for Membrane Elution**

The existing protocol passes 1200mL of PBST wash from the sponge filters through the membrane. Therefore, this part of the work aimed to investigate how this volume of eluent spiked with cysts being passed through the membrane would affect recovery of cysts using megasonic sonication. Results are shown in Table 2 and Figure 3B. In this method of operation the standard procedure offered higher recovery rates of both intact cysts (69% versus 55%) and total count (78% versus 55%) and the megasonic approach appeared more variable. However, greater cyst damage was observed with the standard approach (9% of the total count being shells for the standard method compared with 0% when using megasonic elution). This result suggests that, despite a benefit being observed in terms of lower levels of cyst destruction, megasonic elution would need further optimisation before it could replace the traditional process in terms of the membrane elution stage as overall recovery rates are lower for the megasonic approach.

***Giardia duodenalis* Direct Membrane Seeding**

While the existing procedure operates with 1200mL of PBST and does not allow the membrane to become dry, an alternative approach to study elution of the membranes was also employed. The first reason was to determine whether an alternative method could overcome the poor recovery observed above in the membrane elution stage. The second reason was to investigate the ability of megasonic elution to effectively release potentially embedded cysts from the membrane without damage. The third, motivated by Kerrouche *et al*, was to explore whether smaller elution volumes could be achieved enabling removal of the centrifugation step of the overall protocol and facilitating full automation of the procedure.

Sonication of the membranes seeded directly with cysts showed highly promising results, notably with regards to the difference between standard and megasonic cyst shell levels (Table 3 and Figure 3C). Recoveries from both methods are comparable (at around 62%) when including cyst shells within the total with the megasonic process demonstrating a slightly higher recovery (64% versus 61%) and more reproducibility within the data (standard deviation of 3 compared to 7). However, if cyst shells are not to be included, the megasonic procedure offers a much greater recovery of cysts; specifically, the recovery rates of intact cysts are 28% for the standard method and 63% for the megasonic elution, which is a statistically significant difference ($p=0.007$ using a One-Way ANOVA).

Here, a control was also included to ensure that the centrifugation step was not responsible for producing the damaged cysts in the standard process. It was found that cysts were similarly as damaged following the standard process when the

centrifugation step was skipped (41% of total recovery being shells), suggesting that the elution process itself was the cause of the damage.

The data suggests that megasonic elution is a gentler procedure than manual processing of filters resulting in a lower level of cyst damage, which is advantageous in terms of accurate reporting, utilising recovered cysts in further detection procedures and enabling dual recovery of *Giardia* cysts alongside *Cryptosporidium* oocysts. Furthermore, the volume eluted from the membranes in this step was just 10mL and therefore the sample could proceed directly to the immunomagnetic processing step, avoiding the time-consuming and difficult to automated centrifugation step.

Additionally, when comparing the results of this procedure (direct membrane seeding versus use of 1200mL spiked PBS) the difference in recovery rates, using the intact cyst data, is relatively small. 69% recovery was achieved in the 1200mL experiment for standard recovery compared with 63% here (direct seeding) for megasonic elution. This difference was not found to be a significant difference ($p=0.134$ using a One-Way ANOVA) which implies the traditional procedure could potentially be adapted to trap all cysts onto the membrane, running the sample dry. This would allow a lower elution volume while still retaining recovery rates at an acceptable level, although care would need to be taken to prevent membrane tearing and sample loss using this method.

Effect of Megasonic Waves on *Giardia duodenalis* Cyst Viability

Investigations into how live cysts are affected by megasonic waves revealed that there was a significant difference between the megasonic exposed samples and to the controls, with megasonic samples having somewhat decreased cyst viability compared to the non-exposed samples. Samples both exposed and not exposed to megasonic waves on day 1 had a mean of 81.5 and 84 viable cysts, respectively, which was not significantly different ($p=0.722$). However when comparing both samples on day 7 megasonic exposed and non-exposed cysts, had a mean viability of 64 and 74.5, respectively, which was found to be significantly different ($p=0.041$).

Although there were noted to be significant reductions in the viability of the samples after 1 week of exposure to the megasonic waves when compared to the controls, samples on the day of exposure were not significantly different. The small loss of viability seen one week from date of megasonic exposure wouldn't however be a large issue, as real world water samples should be prepared by the water laboratory as soon as possible as recoveries will decline with increased time. Furthermore the fact that the cysts appear to be less damaged/broken during elution from filter materials outweighs this higher percentage of non-viable cysts from megasonic exposure as they can still be used for DNA based work as well as identified microscopically.

A Complete Procedure of Giardia duodenalis Elution using Megasonic Sonication

Work with the sponges showed that megasonic elution offered comparable recovery rates and the use of fully concentrating the sample onto the membrane offered good recovery rates with megasonic use and the possibility to avoid the centrifugation step as the membrane elution utilised just 10mL. Therefore, a complete procedure of filtration sample processing was compared: the two conditions were firstly, using the standard method, including centrifugation, and secondly, using megasonic elution for both the sponges and the membrane skipping the centrifugation step. Results are shown in Table 4 and Figure 3D. Work by Kerrouche *et al* (2015) achieved comparable recoveries to the standard method when megasonic sonication was employed on *Cryptosporidium* oocyst in elution from filters. This shows that it is possible, at least for *Cryptosporidium*, to optimise the megasonic protocol effectively with regards to parasite filter elution. At present the Giardia recovery rates achieved were lower in the megasonic case, with 55% achieved for intact cysts using megasonic elution compared to 66% with the standard approach. However, again no shells were observed with the megasonic samples whereas around 4% of the cysts were destroyed with the traditional method. Further work would be interesting to assess the efficiency of megasonic elution on field samples. The exposure of cysts to environmental conditions could weaken the cyst shell and cause them to be more likely be damaged during the standard elution protocol, meaning megasonic elution may be a more useful method in this situation.

In the final (see table 4) experiment filter sponges were noted to have expanded in both the megasonic exposed and standard control experiments. This was seen to create a large increase in recovery of cysts from the samples alone (see tables 1 & 4). In the final experiments additional wash steps were introduced into the megasonic methodology in an effort to increase recovery values. These washes focusing on recovering cysts potentially lost by adhering to plastics or being left behind in the plastic bags. The addition of these wash steps would have most likely been involved in the increased recovery of the megasonic method (compared to the previous attempt (table 1)) however the impact of this is difficult to quantify due to the differences in the sponge expansion between experiments having an impact on recoveries.

It should be noted that gamma irradiated cysts were utilized for this work due to the counted reliability in the numbers of cysts inside the suspensions (100 cysts +/- 2). A difference between gamma-irradiated and live *Giardia duodenalis* cyst shell dynamics could potentially affect recoveries when compared to other studies using live cysts. On consulting TCS Biosciences, the distributor of the EasySeed cysts in the UK, knowledge of how well the irradiated cysts represent live parasites during filtration is unknown. For this reason both field and laboratory trials using live cysts and megasonic elution would be useful. Previous work has highlighted that the ability of the GC-Combo Dynabead kit for IMS of *Cryptosporidium* and *Giardia* oo/cysts is not affected by the

viability of the parasites and so this can be negated as a source of difference between live and inactivated cysts (McCuin *et al*, 2001).

Megasonic Elution in Automated Set-Ups with Different Filter Types

The above data has confirmed that megasonic elution offers a potentially viable method for reduction of steps in the standard protocol for *Giardia* monitoring and ensuring intact cysts are recovered. Together with previous data (Kerrouche, 2015) supporting the use of megasonic elution with *Cryptosporidium* this suggests that megasonic elution has the potential for facilitating the creation of automated monitoring systems. In order to explore the potential of megasonic elution with different filter types experiments were undertaken using two different set-ups. A dead-end filtration system was used with the Rexeed 25AX filter and a tangential flow set-up was used with a Fresenius FX1000 filter. Filter types were selected utilising data from previous literature (Wohlsen, 2004). The dead-end set-up has now been fully automated and details will be published elsewhere. The tangential flow system was part of a fully automated system to replace the *Cryptosporidium* regulatory monitoring procedure described earlier, developed by Shaw Water. For the study standard operating procedures were compared with use of the megasonic transducer (during the backflush phase for the dead-end filter and throughout operation of the tangential set-up).

Results are shown in Figure 4. For the dead-end filtration system with the Rexeed filter recovery rates were 31% in the standard procedure and this increased to 49% with the use of megasonic elution (p=0.016). A similar effect was observed in the tangential flow set-up where recovery rates increased from 26% to 37% (p=0.026) when adding in the megasonic sonication steps. Both increases were statistically significant. Interestingly, with these different filter types, megasonic use has an impressive impact at increasing recovery rate, in addition to the other advantages of megasonic operation that have already been discussed. This suggests that systems for waterborne protozoa monitoring should consider the inclusion of megasonic elution and different filter types should be explored to discover the best combination before fully automated systems incorporating megasonic transducers are developed.

Table 1 – Results of Standard and Megasonic Techniques used on Seeded FiltaMax Sponges

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Normal	1	25	21	4	21.0%	25.0%	16.0%
	2	21	17	4	17.0%	21.0%	19.0%
	3	23	21	2	21.0%	23.0%	8.7%
	Mean	23	20	3	19.7%	23.0%	14.6%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	15	15	0	15.0%	15.0%	0.0%
	2	17	17	0	17.0%	17.0%	0.0%
	Mean	16	16	0	16.0%	16.0%	0.0%

Table 1 - The results from these experiments show that the megasonic method is not currently as effective using the current protocol compared to the standard technique. Higher recoveries were achieved using the standard technique. Only two replicates were reported for the megasonic processing as one was lost during the processing. It can be seen however that the megasonic did perform well compared to the standard process if only intact cysts are reported. Here we see that the megasonic process yielded a mean of 16% compare to the mean of 20% for the standard process. As the megasonic process has yet to be fully optimised and is in its infancy this shows promise for its use. Were appropriate totals are rounded to the closest cyst.

Table 2 – Results of Standard and Megasonic Techniques Used on Seeded Membranes with an Increased Eluent Volume

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	78	72	6	72%	78.0%	7.7%
	2	84	71	13	71%	84.0%	15.5%
	3	73	65	8	65%	73.0%	11.0%
	Mean	78	69	9	69%	78.3%	11.4%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	47	47	0	47%	47.0%	0.0%
	2	48	48	0	48%	48.0%	0.0%
	3	69	69	0	69%	69.0%	0.0%
	Mean	55	55	0	55%	54.7%	0.0%

Table 2 – The results of the experiments performed using an increased volume of PBST (1200ml). It can be seen that although the number of cysts recovered by the megasonic technique are not as high as the standard method (mean total number of cysts + shells by megasonic method is 55, compared to 69 by standard method) there is a significant difference between the shell levels recovered from both methods. The megasonic method had 0 shells recovered, however the standard method had a mean of 9 shells recovered. Where appropriate totals have been rounded down to the closest cyst.

Table 3 – Results of Standard and Megasonic Techniques used on Directly Seeded Membranes.

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	64	25	39	25.0%	64.0%	60.9%
	2	53	19	34	19.0%	53.0%	64.2%
	3	66	41	25	41.0%	66.0%	37.9%
	Mean	61	28	33	28.3%	61.0%	54.3%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	61	59	2	59.0%	61.0%	3.3%
	2	67	67	0	67.0%	67.0%	0.0%
	3	64	64	0	64.0%	64.0%	0.0%
	Mean	64	63	1	63.3%	64.0%	1.1%

Table 3 – Recoveries of FiltaMax membranes seeded with *G.duodenalis* cysts using megasonic and standard methodologies. It can be seen that the megasonic methodology has a significant advantage with regards to recovering intact cysts. The total numbers of cysts in both cases are comparable, however the numbers of damaged cysts within the normal method was observed to be over 50%, with the numbers of intact cysts averaging 28% in the standard method replicates, compared to 63% using the megasonic method. Where appropriate totals have been rounded to the closest cyst.

Table 4 - Complete Procedure of *Giardia Duodenalis* Elution using Megasonic Sonication and controls

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Standard	1	74	72	2	72.0%	74.0%	2.7%
	2	73	66	7	66.0%	73.0%	9.6%
	3	64	60	4	60.0%	64.0%	6.3%
	Mean	70	66	4	66.0%	70.3%	6.2%

	Replicate	No. of Cysts (Cysts + Shells)	No. of Intact Cysts	No. of Shells	Intact Cyst Recovery (%)	Recovery of Cysts + Shells (%)	Ratio of Shells to Cysts (%)
Megasonic	1	50	50	0	50.0%	50.0%	0.0%
	2	56	56	0	56.0%	56.0%	0.0%
	3	58	58	0	58.0%	58.0%	0.0%
	Mean	55	55	0	54.7%	54.7%	0.0%

Table 4 – The results of this experiments show that the megasonic methodology still requires optimisation before it can match the recoveries achieved using the standard method of elution. The recoveries achieved from the megasonic sonication experiments are however acceptable and still managed to recover a significant number of cysts from the sample, all of which were found to be intact. This was not the case with the standard method and on average around 6% of the recovered objects were shells. Where appropriate totals have been rounded down to the closest cyst.

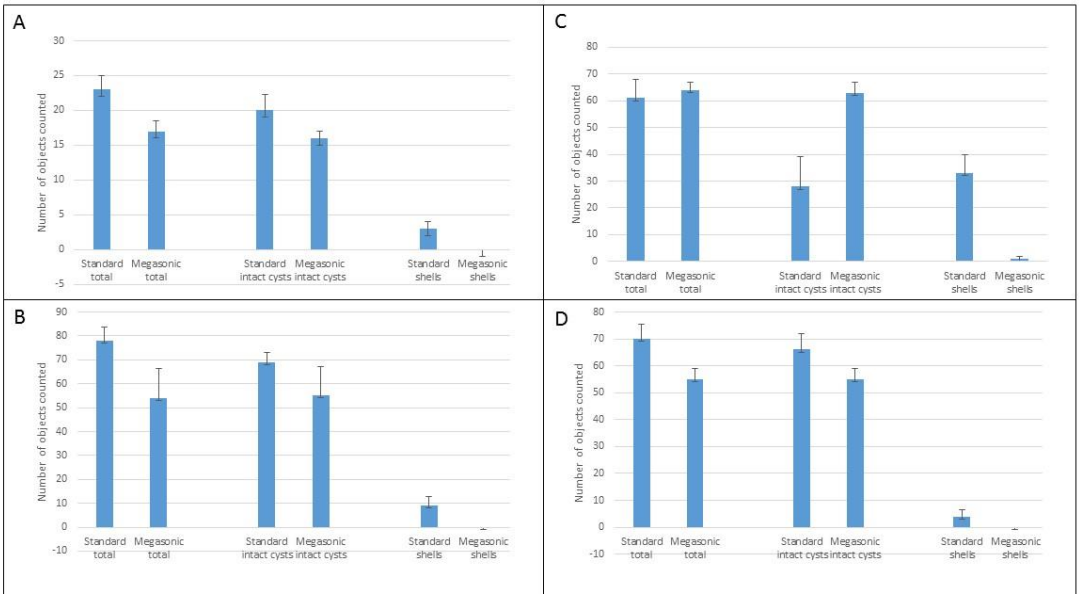


Figure 3 – A) B) A graph of recoveries from standard and megasonic experiments using 1200mL of eluent through the membrane. From the graph it can be seen that the megasonic method was not able to recover a similar amount of cysts when compared to the standard method in this experiment (mean 69 intact cysts by standard method vs mean 55 cysts by megasonic method). There were however an increased number of damaged cysts in the standard method, compared to the megasonic method in which there were none (standard method had a mean of 9 shells while megasonic had 0). **C)** A graph of cyst recoveries from the direct seeding experiments. It can be seen that a comparable level of mean total objects were recovered by both the standard and the megasonic methodologies (mean 64 cysts by Megasonic vs mean 61 cysts by Standard). However the mean numbers of intact cysts in the standard method was significantly less in the standard method (mean 28 intact cysts) which had an increased number of shells (mean 33 by standard method vs mean 1 by megasonic method) when compared to the megasonic method (mean 63 intact cysts). **D)** A graph showing the recoveries found using a complete methodology for megasonic elution against the control standard methodology. It can be seen that the standard methodology achieved higher recoveries of both cysts plus shells (mean of 70 using standard method compared to a mean of 55 using the megasonic method) and also cysts (mean of 66 using standard method compare to a mean of 55 using megasonic method) discounting shells. There were a small number of shells observed within the standard methodology samples which were not seen in the megasonic methodology (mean of 4 shells in the standard method).

Table 5: Recovery rates for the megasonic protocols.

Filter	**Tangential flow filter**		**Dead-end flow filter**	
Recovery rate (%)	Without megasonic	With megasonic	Without megasonic	With megasonic
Test1	25	53	24	41
Test 2	28	50	29	33
Test 3	39	43	26	38
Average	30.66	48.66	26.33	37.33

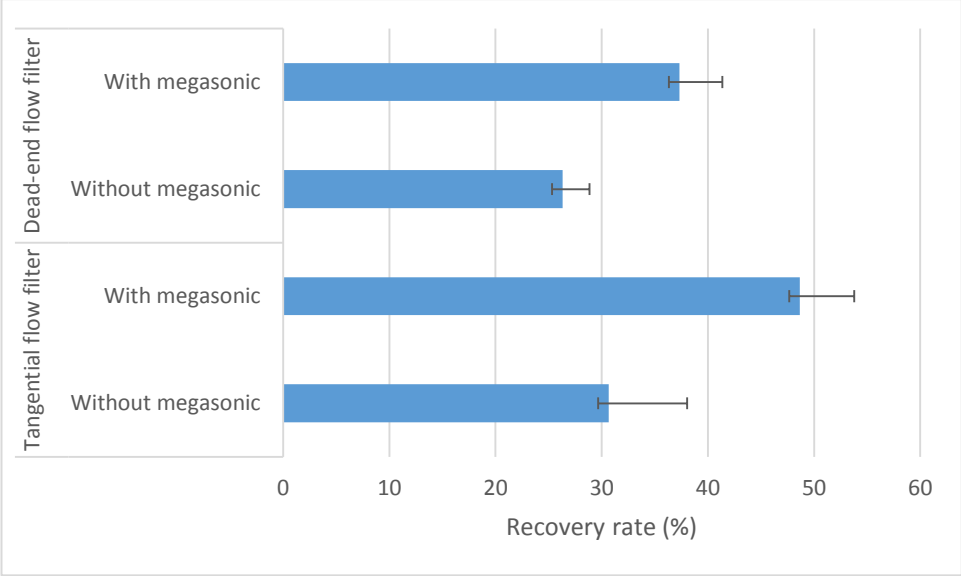


Figure 4. Percentage recovery rate for *Cryptosporidium* in two different filtration set-ups. The dead-end flow filter used a Rexeed 25XA filter and shows an increase from 31 to 49% when adding in megasonic elution. The tangential flow set-up used a Fresenius FX1000 and also demonstrates an increase in recovery rate, this time from 26 to 37%, with megasonic use incorporated.

Conclusions

The data presented in this article demonstrates that megasonic elution offers an effective means to elute protozoan parasites from filtration set-ups. With *Cryptosporidium* and filter types, such as Rexeed and Fresenius, higher recovery rates were observed using megasonic sonication. With the use of megasonics, recovery rates were increased by around 50% in both cases. The filter types were operated in a dead-end and tangential flow set-up, respectively, indicating that megasonic use can be beneficial in both types of operation.

For *Giardia duodenalis*, recovery rates from the sponge stage of filtration were fairly similar whether the standard or megasonic approach was used, and this step represented the greatest loss in the procedure. The direct seeding of the membrane showed excellent recovery rates with megasonic use, more than doubling the recovery of intact cysts, revealing the ability of megasonic elution to effectively release potentially embedded cysts from the membrane without damage. However, the current protocol passes 1200mL of PBST wash from the sponge filters through the membrane and does not allow the membrane to become dry, and here recovery rates were lower. Similar data showing slightly lower recovery rates were observed comparing a full standard protocol with a megasonic elution of both sponges and membranes, where the latter offered the advantage of skipping the centrifugation step.

Overall, recovery rates for *Giardia* with megasonic elution have not been found to be as high as compared to the well optimized, internationally used FiltaMax system. However, this could potentially be improved with further optimisation or perhaps switching to different filter systems is essential to maximise the benefits of megasonic elution. Advantages to the megasonic approach include the reduction in manual labour required to process a filter, the lack of centrifugation required for the method and, especially for *Giardia*, the seemingly ‘gentler’ elution process, resulting in lower levels of cyst damage. Additionally, the final data shown for *Cryptosporidium* with other filters demonstrates an increase in recovery with the use of megasonic sonication suggesting that a combination of the correct filter type with megasonic use could combine the aforementioned advantages with enhanced recovery rates. Such a system would also have the potential for effective recovery of both *Cryptosporidium* and *Giardia*, with minimal damage, and offered automated sample processing. Future work will explore the development and validation of this approach.

Achnowledgements:

The authors would like to acknowledge Moredun Scientific and Aquavalens for funding to make this work possible.

References

- Baldursson, S. & Karanis, P. (2011) Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2004-2010. Water Research. 45: pp6603-6614.
- Barwick, R. S., D. A. Levy, G. F. Braun, M. J. Beach, & R. L. Calderon. (2000) *Surveillance for water-borne disease outbreaks—United States*, *1997–1998*. Morbidity and Mortality Weekly Report, CDC Surveillance Summary : 49(SS-4): pp1–36.
- Cacciò, M.C. & Sprong, H (*Eds. Luján, H.D. & Svärd, S.)* (2011) Chapter 2 - *Epidemiology of giardiasis in Humans.* In: Giardia – A Model Organism: pp17.
- Chitra, S., Paramasivan, K., Sinha, P. & Lal, K. (2004) *Ultrasonic treatment of liquid waste containing EDTA.* Journal of cleaner production 12(4): pp429–435.
- Feng, Y & Xiao, L (2011) *Zoonotic potential and molecular epidemiology of Giardia species and giardiasis.* Clinical Microbiology Review, 24: pp110 – 140.
- Ferguson, C., Kaucner, C., Krogh, M., Deere, D. & Warnecke, M. (2004) *Comparison of methods for the concentration of Cryptosporidium oocysts and Giardia cysts from raw waters.* Canadian Journal of Microbiology, 50(9): 675-682.
- Idexx (2016) Filta-Max Filter Modules from IDEXX Company. http://www.idexx.co.uk/resource-library/water/filta-max-procedure.pdf (Last accessed 21/02/2016).
- Jakubowski, W. (1988) *Purple burps and the filtration of drinking water supplies.* American Journal of Public Health; 78: pp123–125.
- Julio, C., Vilares, A., Oleastro, M., Ferreira, I., Gomes, S., Monterio, L., Nunes, B., Tenreiro, R. & Angelo, H. (2012) *Prevalence and risk factors for Giardia duodenalis infection among children: A case study in Portugal.* Parasites & Vectors (5)22; pp 1-8.
- Kerrouche, A., Desmulliez, M.P.Y., & Bridle, H. (2015) Megasonic sonication for cost-effective and automatable elution of Cryptosporidium from filters and membranes. Journal of Microbial Methods.118:pp123–127.
- Lake, I.R., Nichols, G., Bentham, G., Harrison, F.C., Hunter, P.R. & Kovats, S.R. (2007) *Cryptosporidiosis decline after regulation, England and Wales, 1989–2005*. Emerging Infectious Disease. 13: pp623–625
- Lane, S. & Lloyd, D. (2002) *Current trends in research into the waterborne parasite Giardia.* Critical Reviews in Microbiology. 28 (2): pp123-147.
- McCuin, R.M., Bukhari, Z., Sobrinho, J. & Clancy, J.L. (2001) *Recovery of Cryptosporidium oocysts and Giardia cysts from source water concentrates using immunomagnetic separation.* Journal of Microbial Methods; 45: 69-76.
- McCuin, R.M. & Clancy, J.L. (2003) Modifications to United States Environmental Protection Agency Methods 1622 and 1623 for detection of Cryptosporidium oocysts and Giardia cysts in water. Applied and Environmental Microbiology, 69(1):267-274.

- Method 1623.1, 2012. Method 1623.1: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. Environmental Protection Agency, United States.
- Porter, J.D., Gaffney, C., Heymann, D and Parkin, W. (1990) *Food-borne Outbreak of Giardia lamblia*. American Journal of Public Health, 80(10): pp1259-1260.
- Robertson & Lim (*Eds. Luján, H.D. & Svärd, S.)* (2011a) Chapter 3 – *Waterborne and Environmentally-Borne Giardiasis.* In: Giardia – A Model Organism: pp29.
- Robertson & Lim (*Eds. Luján, H.D. & Svärd, S.)* (2011b) Chapter 3 – *Waterborne and Environmentally-Borne Giardiasis.* In: Giardia – A Model Organism: pp55.
- Robertson & Lim (*Eds. Luján, H.D. & Svärd, S.)* (2011c) Chapter 3 – *Waterborne and Environmentally-Borne Giardiasis.* In: Giardia – A Model Organism: pp43.
- Robertson & Lim (*Eds. Luján, H.D. & Svärd, S.)* (2011d) Chapter 3 – *Waterborne and Environmentally-Borne Giardiasis.* In: Giardia – A Model Organism: pp44.
- Roxstrom-Lindquist, K., Palm, D., Reiner, D., Ringqvist, E. & Svard, S.G. (2006) Giardia immunity – an update. TRENDS in Parasitology, 22(1) pp26-31.
- Smith, H.W., Cacciò, S.M., Cook, N., Nichols, R.A.B & Tait, A. (2007) *Cryptosporidium and Giardia and foodborne zoonoses.* Veterinary Parasitology, 149: pp29-40.
- Sonosys (2016) The megasonic transducer as purchased from Sonosys Company. (http://www.sonosys.de/products/submersible-transducer (Last accessed 21/02/2016).
- Sprong, H. Cacciò, S.M. & van der Giessen, J.W.B. (2009) *Identification of Zoonotic Genotypes of Giardia duodenalis.* PLoS Neglected Tropical Diseases, 3 (12): pp1-12.
- Stuart, J. M., H. J. Orr, F. G. Warburton, S. Jeyakanth, C. Pugh, I. Morris, J. Sarangi, & G. Nichols (2003). *Risk factors for sporadic giardiasis: a case-control study in southwestern England*. Emerging Infectious Diseases, 9: pp229–233.
- Thompson, A.R.C. (2000) *Giardiasis as a re-emerging infectious disease and its zoonotic potential.* International Journal for Parasitology. 30: pp1259-1267.
- UK Environment Agency (2010). Methods for the isolation, identification and enumeration of Cryptosporidium oocysts and Giardia cysts. The United Kingdom Environment Agency Blue Book publications. Microbiology of Drinking Water: Part 14).
- Waters, E.K., Hamilton, A.J., Sidhu, H.S., Sidhu, L.A. & Dunbar, M. (2016) *Zoonotic Transmission of Waterborne Disease: A Mathematical Model.* Bulletin of Mathematical Biology, 78: pp169-183.
- Water Supply (Water Quality) Regulations 1999, SI No. 1524.
- Wohlsen, T., Bates, J., Gray, B. & Katouli, M (2004) *Evaluation of Five Membrane Filtration Methods for Recovery of Cryptosporidium and Giardia Isolates from Water Samples*. Applied and Environmental Microbiology, 70 (4): pp2318-2322.

5.4 Appendix D: Supplementary Information

5.4.1 Images of *G. duodenalis* cysts

FITC stain

Pear-shape cyst.

DAPI stain

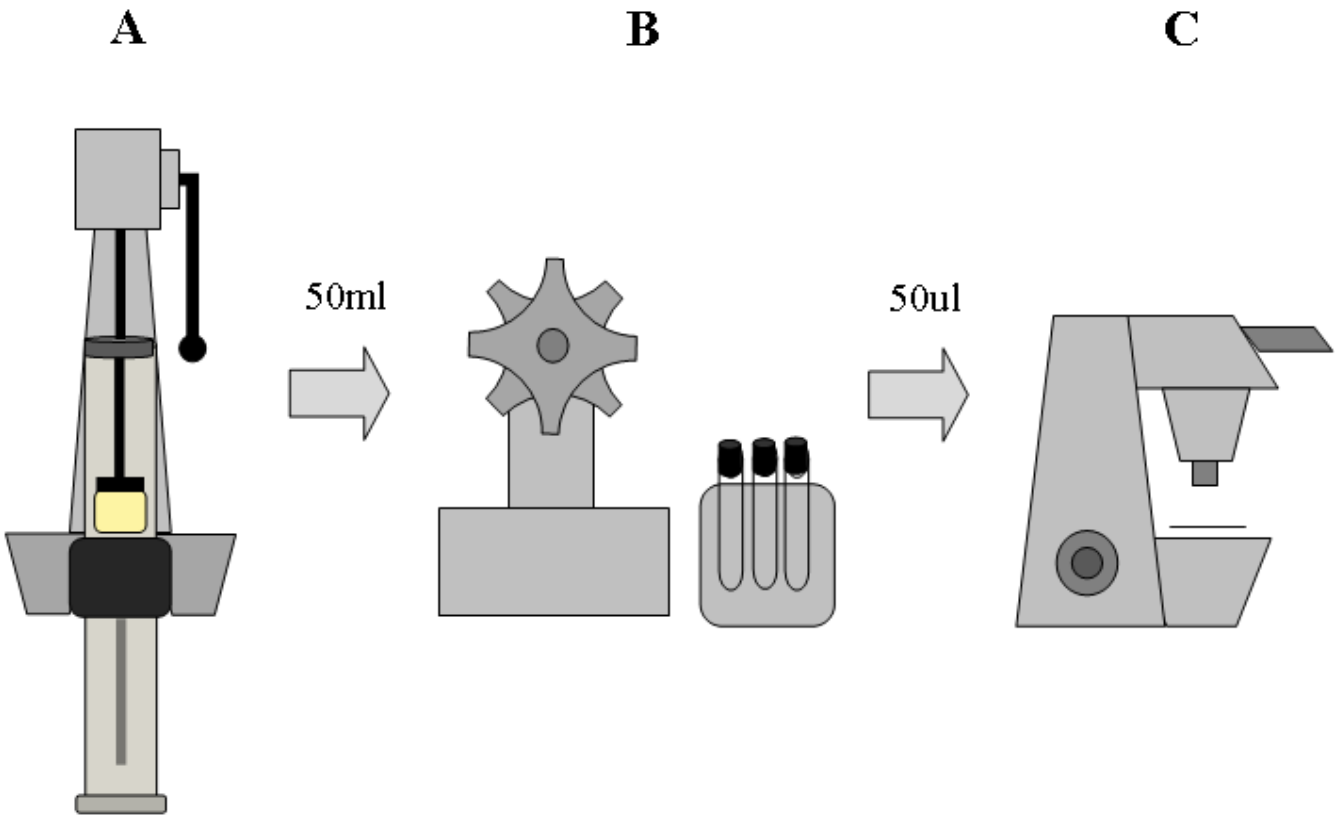
2 - 4 trophozoite nuclei may be visible.

DIC microscopy

The nuclei, remnants of the flagellar axonemes and the median body should be visible.

The above images show *G. duodenalis* cysts viewed under both ultraviolet (UV) and DIC (Direct Interference Contrast) microscopy. The first image (from top to bottom) is of a *G. duodenalis* cyst stained with FITC-CmAb (fluorescein isothiocyanate-labeled anti-*Giardia* cyst antibodies) to which allows visualisation of the cyst by an apple green glow being visualised under UV light exposure. The second photo is of the same cyst however shows DAPI (4',6-diamidino-2-phenylindole) staining of the nuclei within the cyst body. The final image shows visualisation of the cyst using DIC microscopy, which allows the internal contents of the cyst to be seen with various typical structures observed within. Image by John Watkins - CREH Labs (UK Environment Agency, 2010).

5.4.2 Diagram of Filta-Max™ Standard Methodology



This diagram shows the three main stages of processing and analysis of a water sample in terms of investigating a water supply for presence of *G. duodenalis* using the Filta-Max™ methodology. To begin a sample of the water is taken and passed through a filter module, which traps any *G. duodenalis* present in the sample, embedding them within the filter foam discs within (not shown in this diagram). The filter module is then expanded and washed using eluting fluid via a wash station apparatus (A) which removes the parasite from the filter and suspends it within the wash fluid. This fluid is then filtered through a membrane allowing reduction of sample volume (resulting in a total of 50ml). This sample is then centrifuged and processed using Immuno-magnetic Separation (B), which uses magnetic beads coated in antibodies specific for *G. duodenalis*, causing them to adhere to the beads. These beads are then removed from the fluid using various magnet to result in a further reduced final liquid volume of 50ul containing the parasite. This sample is then transferred onto a slide and stained to allow identification under UV light, which allows an trained analyst to enumerate the recovered parasite from the sample (C).

6. Thesis References

- Abeywardena, H., Jex, A.R., Nolan, M.J., Haydon, S.R., Stevens, M.A., McAnulty, R.W. & Gasser, R.B. (2012) *Genetic characterisation of Cryptosporidium and Giardia from dairy calves: discovery of species/genotypes consistent with those found in humans*. Infection, Genetics and Evolution, 12: pp1984–1993.
- Adam, R.D. (2001). *Biology of Giardia lamblia.* Clinical Microbiology Reviews, 14: pp447-475.
- Alexander, C., Jones, B., Inverarity, D. & Pollock, K.G.J. (2014) *Genotyping of Giardia isolates in Scotland: a descriptive epidemiological study.* Epidemiology and Infection, 142: pp1636-1639.
- Alexander, C.L., Niebel, M. & Jones, B. (2013) *The rapid detection of Cryptosporidium and Giardia species in clinical stools using the Quik Chek immunoassay*. Parasitology International, 62: pp552-553.
- Amar, C.F.L., Dear, P.H., Pedra-Diaz, S., Looker, N., Linnane, E. & McLauchlin, J. (2002) *Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of Giardia duodenalis in human feces.* Journal of Clinical Microbiology, 40: pp446–452
- Andrews, R.H., Adams, M., Boreham, P.F., Mayrhofer, G. & Meloni, B.P. (1989) *Giardia intestinalis: electrophoretic evidence for a species complex.* International Journal for Parasitology, 19: pp183–190.
- Andrews, R.H., Monis, P.T., Ey, P.L & Mayrhofer, G. (1998) *Comparison of the levels of intra-specific genetic variation within Giardia muris and Giardia intestinalis.* International Journal for Parasitology, 28: pp1179–1185
- Ankarklev, J., Jerlström-Hultqvist, J., Ringqvist, E., Troell, K. & Svard, S.G. (2010) *Behind the smile: cell biology and disease mechanisms of Giardia species.* Nature Reviews Microbiology, 8: pp412-422.
- Appelbee, A.J., Frederick, L.M, Heitman, T.L. & Olson, M.E. (2003) *Prevalence and genotyping of Giardia duodenalis from beef calves in Alberta, Canada.* Veterinary Parasitology, 112: pp289-294.

- Baldursson, S & Karanis, P. (2011) *Waterborne Transmission of Waterborne Parasites: Review of Worldwide Outbreaks – An Update 2004-2010*. Water Research 45: pp6603-6614.
- Ballweber, L.R., Xiao, L., Bowman, D.D., Kahn, G. & Cama, V.A. (2010) *Giardiasis in dogs and cats: update on epidemiology and public health significance.* TRENDS in parasitology, 26: pp180-189.
- Bartelt, L.A. & Sartor, R.B. (2015) *Advances in understanding Giardia: determinants and mechanisms of chronic sequelae*. F1000Prime Reports, 7: pp62.
- Barwick, R. S., D. A. Levy, G. F. Braun, M. J. Beach, & R. L. Calderon. (2000) *Surveillance for water-borne disease outbreaks—United States, 1997–1998. Morbidity and Mortality Weekly Report,* CDC Surveillance Summary: 49(SS-4): pp1–36.
- Benchimol, M. & De Souza, W. (2011g) *The Ultrastructure of Giardia During Growth and Differentiation.* In: Lujam. H.D. & Svärd, S. (Eds) *Giardia: A model organism.* SpringerWeinNewYork. Pp142.
- Birky, C.W. (2010) *Giardia sex? Yes, but how and how much?* Trends in Parasitology, 26: pp70-74.
- Blanchard, R. (1888) *Remarques sur le megastome intestinal*. Bulletin de la Société Zoologique de France; 30, pp18–19.
- Van den Bossche, D., Cnops, L., Verschueren, J. & Van Esbroeck, M. (2015) *Comparison of four rapid diagnostic tests, ELISA, microscopy and PCR for the detection of Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica in feces.* Journal of Microbiological Methods, 110: pp78-84.
- Bouzid, M., Halai, K., Jeffreys, D. & Hunter, P.R. (2015) *The prevalence of Giardia infection in dogs and cats, a systematic review and meta-analysis of prevalence studies from stool samples.* Veterinary Parasitology, 207: pp181-202.
- Breathnach, A.S., McHugh, T.D. & Butcher, P.D. (2010) *Prevalence and clinical correlations of genetic subtypes of Giardia lamblia in an urban setting.* Epidemiology and Infection, 138: pp1459–1467.
- Buchel, L.A., Gorenflot, A., Chochillon, C., Savel, J. & Gobert J.G. (1987) *Invitro excystation of Giardia from humans: a scanning electron microscopy study.* The Journal of Parasitology, 73: pp487-493.

- Cacciò, S.M., Beck, R., Lalle, M., Marinculic, A. & Pozio, E. (2008) *Multilocus genotyping of Giardia duodenalis reveals striking differences between assemblages A and B.* International Journal for Parasitology, 38: pp1523-1531.
- Cacciò, S.M. & Ryan, U. (2008) *Molecular epidemiology of giardiasis.* Molecular and Biochemical Parasitology, 160: pp75–80.
- Cacciò, S.M. & Sprong, H. (2011) *Epidemiology of Giardiasis in Humans*. In: Lujan, H.D & Svard, S. (Eds) *Giardia – A Model Organism*, SpringerWienNewYork, pp18.
- Cacciò, S.M. & Sprong, H. (2011c) *Taxonomy of Giardia Species*. In: Lujam. H.D. & Svärd, S. (Eds) *Giardia - A model organism.* SpringerWeinNewYork. Pp24.
- Cacciò, S.M. & Sprong, H. (2011d) *Taxonomy of Giardia Species*. In: Lujam. H.D. & Svärd, S. (Eds) *Giardia - A model organism*. SpringerWeinNewYork. Pp25.
- Cacciò, S.M., Thompson, A.R.C., McLaughlin, J. & Smith, H.V. (2005) *Unravelling Cryptosporidium and Giardia Epidemiology.* TRENDS in Parasitology, 21: pp430-437.
- Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Correia Da Costa, J.M. & Mezo, M. (2009) *Detection of Cryptosporidium spp. And Giardia duodenalis in surface water: A health risk for humans and animals.* Water Research, 43: pp4133-4142.
- Cebra, C.K., Mattson, D.E., Baker, R.J., Sonn, R.J. & Dearing, P.L. (2003). *Potential pathogens in feces from unweaned llamas and alpacas with diarrhea.* Journal of the American Veterinary Medical Association, 223: pp1806–1808.
- Chitra, S., Paramasivan, K., Sinha, P. & Lal, K. (2004) *Ultrasonic treatment of liquid waste containing EDTA.* Journal of Cleaner Production, 12: pp429–435.
- Clayton, R. & Waite, M. (2012) *A Review of Current Knowledge: Giardia in Water Supplies.* Foundation for Water Research (http://www.fwr.org/giardia.pdf). (Accessed Oct2015).
- Coklin, T., Farber, J., Parrington, L. & Dixon, B. (2007) *Prevalence and molecular characterization of Giardia duodenalis and Cryptosporidium spp. in dairy cattle in Ontario, Canada.* Veterinary Parasitology, 150: pp297–305.
- Cooper, M.A., Adam, R.D., Worobey, M. & Sterling, C.R. (2007) *Population genetics provides evidence for recombination in Giardia*. Current Biology, 17: pp1984-1988.

- Daly, E.R., Roy, S.J., Blaney, D.D., Manning, J.S., Hill, V.R., Xiao, L. & Stull, J.W. (2010) *Outbreak of giardiasis associated with a community drinking-water source.* Epidemiology and Infection, 138: pp491-500.
- D'Anchino, M., Orlando, D. & De Feudis, L. (2002). *Giardia lamblia infections become clinically evident by eliciting symptoms of irritable bowel syndrome.* Journal of Infection, 45: pp169–172.
- Dawson, S.C., Nohýnková, E. & Cipriano, M (2011) *Cell Cycle Regulation and Cell Division in Giardia.* In: Lujam. H.D. & Svärd, S. (Eds) *Giardia - A model organism*. SpringerWeinNewYork. Pp163.
- Dixon, B., Parrington, L., Cook, A., Pintar, K., Pollari, F., Kelton, D. & Farber, J. (2011*) The potential for zoonotic transmission of Giardia duodenalis and Cryptosporidium spp. from beef and dairy cattle in Ontario, Canada*. Veterinary Parasitology, 175: pp20-26
- Dobell, C. (1920). *The discovery of the intestinal protozoa of man.* Proceedings of the Royal Society of Medicine, 13: pp1–15.
- Dubey, J.P., Miller, N.L. & Frenkel, J.K (1970) *The Toxoplasma gondii oocysts from cat feces.* The Journal of Experimental Medicine, 132: pp636-662.
- Dubey, J.P. (1998) *Toxoplasma gondii oocysts survival under defined temperatures. The Journal of Parasitology*, 84: pp862-865.
- DWI Report 70-2-155 (2003) The Enumeration of Giardia in Drinking Water. Available at: http://www.dwi.gov.uk/research/completed-research/reports/DWI70-2-155_giardia.pdf [Accessed 16 Dec. 2015].
- Esch, K.J. & Peterson, C.A (2013) *Transmission and Epidemiology of Zoonotic Protozoal Diseases of Companion Animals*. Clinical Microbiology Reviews, 26: pp58-85.
- Ekdahl, K. & Andersson, Y. (2005) *Imported Giardiasis: Impact of International Travel, Immigration, and Adoption*. The American Journal of Tropical Medicine and Hygiene, 72: pp825-830.
- Ehsan, A.M., Geurden, T., Casaert, S., Parvin, S.M., Islam, T.M., Ahmed, U.M., Levecke, B., Vercruysse, J & Claerebout, E. (2015) *Assessment of Zoonotic*

Transmission of Giardia and Cryptosporidium between Cattle and Humans in Rural Villages in Bangladesh. PLos One, 10 (2).

- Elwin, K., Fairclough, H.V., Hadfield, S.J. & Chalmers, R.M. (2014) *Giardia duodenalis typing from stools: a comparison of three approaches to extracting DNA and validation of a probe-based real-time PCR typing assay*. Journal of Medical Microbiology, 63: pp38-44.
- Esch, K.J. & Petersen, C.A. (2012) *Transmission and Epidemiology of Zoonotic Protozoal Diseases of Companion Animals*. Clinical Microbiology Review, 26: pp58-85.
- Escobedo, A.A. & Cimerman, S. (2007) *Giardiasis: a pharmacotherapy review.* Expert Opinion on Pharmacotherapy, 8: pp1885–1902
- Fayer, R., Santin, M., Trout, J.M., DeStefano, S., Koenen, K. & Kaur, T. (2006) *Prevalence of microsporidia, Cryptosporidium spp., and Giardia spp. in beavers (Castor canadensis) in Massachusetts.* Journal of Zoo and Wildlife Medicine, 37: pp492–497.
- Feng, Y. & Xiao, L. (2011) *Zoonotic potential and molecular epidemiology of Giardia species and giardiasis.* Clinical Microbiology Reviews, 24: pp110–140.
- Filice, F.P. (1952) *Studies on the cytology and life history of a Giardia from the laboratory rat.* University of California Publcations in Zoology, 57: pp53–146.
- Gaafar, M.R. (2011) *Evaluation of enzyme immunoassay techniques for diagnosis of the most common intestinal protozoa in fecal samples.* International Journal of Infectious Diseases, 15: pp541-544.
- Gardner, T.B. & Hill, D.R. (2001) *Treatment of Giardiasis.* Clinical Microbiology Reviews, 14: pp114-128.
- Geurden, T., Vanderstichel, R., Pohle, H., Ehsan, A., von Samson-Himmelstjerna, G., Morgan, E.R., Camuset, P., Capelli, G., Vercruysse, J. & Claerebout, E. (2012*) A multicentre prevalence study in Europe on Giardia duodenalis in calves, with molecular identification and risk factor analysis.* Veterinary Parasitology, 190: Pp383-390.
- Gilmour, R.A., Smith, H.V., Smith, P.G. Morris, G.P. & Girdwood, R.W.A. (1991) *The Occurrence and Viability of Giardia spp. Cysts in UK Waters.* Water Science & Technology, 24: pp179-182.

- Goka, A.K.J., Rolson, D.D.K., Mathan., V.I. & Farthing, M.J.G. (as reviewed by Johnston *et al*, 2003) (1990) *The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 84: pp66-67.
- Gomez-Couso, H., Ortega-Mora, L.M., Aguado-Martinez, A., Rosadio-Alcantara, R., Maturrano-Hernandez, L., Luna-Espinoza, L., Zanabria-Huisa, V. & Pedraza-Diaz, S. (2012) *Presence and molecular characterisation of Giardia and Cryptosporidium in alpacas (Vicugna pacos) from Peru.* Veterinary Parasitology, 187: pp414-420.
- Gov.uk. (2016). Giardia: guidance and data - GOV.UK. Available at: https://www.gov.uk/guidance/giardia [Accessed 10 May 2016].
- Graczyk, T.K., Bosco-Nizeyi, J., Ssebide, B., Thompson, R.C., Read, C. & Cranfield, M.R., (2002). *Anthropozoonotic Giardia duodenalis genotype (assemblage) infections in habitats of free-ranging human-habituated gorillas, Uganda*. Journal of Parasitology, 88: pp905–909.
- Halliez, M.C.M & Buret, A.G (2013) *Extra-intestinal and long term consequences of Giardia duodenalis infections*. World Journal of Gastroenterology, 19: pp 8974-8985.
- Hanevik, K., Hausken, T., Morken, M.H., Strand, E.A., Mørch, K., Coll, P. Helgeland, L. & Langeland, N. (2007) *Persisting symptoms and duodenal inflammation related to Giardia duodenalis infection.* Journal of Infection, 55: pp524–530.
- Hanevik, K., Wensaas, K., Rortveit, G., Eide, G.E., Morch, K. & Langeland, N. (2014) *Irritable Bowel Syndrome and Chronic Fatigue 6 Years After Giardia Infection: A Controlled Prospective Cohort Study*. Clinical Infectious Diseases, 59: pp1394-1400.
- Hoar, B.R., Paul, R.R., Siembieda, J., das Gracas, M., Pereira, C. & Atwill, E.R. *Giardia duodenalis in feedlot cattle from the central and western United States.* BMC Veterinary Research, 5: pp37
- Homan, W.L & Mank. T.G (2001) *Human Giardiasis: genotyped linked differences in clinical symptomatology.* International Journal for Parasitology, 31: pp822-826.
- Hunter, P.R. & Thompson, A.R.C (2005) *The zoonotic transmission of Giardia and Cryptosporidium.* International Journal for Parasitology, 35: pp1181-1190.
- Hps.scot.nhs.uk. (2016). *Giardia - GIZ - HPS*. Available at: http://www.hps.scot.nhs.uk/giz/giardia.aspx [Accessed 16 Apr. 2016].

- Jerlström-Hultqvist, J., Ankarklev, J. & Staffan, S.G. (2010) *Is human giardiasis caused by two different Giardia species?* Gut Microbes 1(6): pp379-382
- Johnston, S.P., Ballard, M.M., Beach, M.J., Causer, L. & Wilkins, P.P. (2003) *Evaluation of Three Commercial Assays for Detection of Giardia and Cryptosporidium Organisms in Fecal Specimens*. Journal of Clinical Microbiology, 41: pp623-626.
- Jones, K. H. & Senft, J. A. (1985). *An improved method to determine cell viability by simultaneous staining with fluorescein diacetate and propidium iodide*. Journal of Histochemistry and Cytochemistry, 331: pp77–79.
- Karanis, P., Kourenti, C. & Smith, H. (2007) *Water-borne transmission of protozoan parasites: a review of world-wide outbreaks and lessons learnt.* Journal of Water and Health, 5: pp1–38.
- Katz, D.E., Heisey-Grove, D., Beach, M., Dicker, R.C. & Matyas, B.T. (2006) *Prolonged outbreak of Giardiasis with two modes of transmission*. Epidemiology and Infection, 134: pp935-941.
- Katzer, F., Canton, G., Burrells, A., Palarea-Albaladejo, J., Horton, B., Bartley, P.M., Pang, Y., Chianini, F., Innes, E.A. & Benavides, J. (2014) *Immunization of lambs with the S48 strain of Toxoplasma gondii reduces tissue cyst burder following oral challenge with a complete strain of the parasite.* Veterinary Parasitology, 205: pp46-56.
- Kerrouche, A., Desmulliez, M.P.Y., & Bridle, H. (2015) *Megasonic sonication for cost-effective and automatable elution of Cryptosporidium from filters and membranes.* Journal of Microbial Methods, 118: pp123–127.
- Khan, S.M., Debnath, C., Pramanik, A.K., Xiao, L., Nozaki, T. & Ganguly, S. (2011). *Molecular evidence for zoonotic transmission of Giardia duodenalis among dairy farm workers in West Bengal, India.* Veterinary Parasitology, 178: pp342–345.
- Koehler, A.V., Jex, A.R., Haydon, S.R., Stevens, M.A. & Gasser, R.B. (2014) *Giardia/giardiasis – A perspective on diagnostic and analytical tools.* Biotechnology Advances, 32: pp280-289.
- Kunstler, J. (1882) *Sur cinq protozoaires parasites nouveaus.* Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales; 95, pp347–349.
- Lambl, W. (1859). *Mikroskopische untersuchungen der Darmexcrete. Vierteljahrsschr. Prakst*, Heikunde, 61: pp1–58.

- Lane, S. & Lloyd, D. (2002). *Current trends in research into the waterborne parasite Giardia*. Critical Reviews in Microbiology, 28: pp123-147.
- Levecke, B., Dorny, P., Geurden, T., Vercammen, F. & Vercruysse, J. (2007). *Gastrointestinal protozoa in non-human primates of four zoological gardens in Belgium.* Veterinary Parasitology, 148: pp236–246.
- Van Lieshout, L. & Roestenberg, M (2015) *Clinical consequences of new diagnostic tools for intestinal parasites.* Clinical Microbiology and Infection, 21: pp520-528.
- Lisle, J.T. and Rose, J.B. (1995) *Cryptosporidium contamination of water in the USA and UK: a mini review*. Journal of Water Supply and Technology-AQUA, 44: pp103-117
- Liu, A., Zhang, X., Zhang, L., Wang, R., Li, X., Shu, J., Zhang, X., Shen, Y., Zhang, W. & Ling, H. (2012). *Occurrence of bovine giardiasis and endemic genetic characterization of Giardia duodenalis isolates in Heilongjiang Province, in the Northeast of China.* Parasitology Research, 111: pp655–661.
- Mark-Carew, M.P., Khan, Y., Wade, S.E, Schaaf, S. & Mohammed, H.O. (2010) *Incidence of and risks associated with Giardia infections in herds on dairy farms in the New York City Watershed.* Acta Veterinaria Scandanavica, 52:44.
- Mayrhofer, G., Andrews, R.H., Ey, P.L. & Chilton, N.B. (1995) *Division of Giardia isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with Giardia muris.* Parasitology, 111: pp11–17
- Meyer, E.A. (1985) *The epidemiology of giardiasi*s. Parasitology Today, 1: pp101–105.
- Mideo, N. (2009) *Parasite Adaptions to Within-host Competition.* Trends in Parasitology 25: pp261-268.
- Miguella, P. M., Wade, S.E., Chang, Y., Schaaf, S. & Mohammed, H.O. (2012) *Prevalence of Giardia duodenalis assemblages among dairy herds in the New York City Watershed.* Veterinary Parasitology, 185: pp151-157.
- Monis, P.T., Andrews, R.H., Mayrhofer, G. & Ey, P.L. (2003). *Genetic diversity within the morphological species Giardia intestinalis and its relationship to host origin*. Infection Genetics and Evolution, 3: pp29–38.

- Monis, P.T., Cacciò, S.M. & Thompson, R.C.A. (2009) *Variation in Giardia: towards a taxonomic revision of the genus.* Trends in Parasitology, 25: pp93-100.
- Monis, P. T., Mayrhofer, G., Andrews, R.H., Homan, W.L., Limper, L. & Ey, P.L (1996) *Molecular genetic analysis of Giardia intestinalis isolates at the glutamate dehydrogenase locus.* Parasitology, 112: pp1–12.
- Nygard, K., Schimmer, B., Sobstad, O., Walde, A., Tveit, I., Langeland, N., Hausken, T. & Aavitsland, P. (2006) *A large community outbreak of waterborne giardiasis – delayed detection in a non-endemic urban area*. BMC Public Health, 6: 141.
- O'Handley, R.M. Cockwill, C., Jelinski, M., McAllister, T.A. & Olson, M.E. (2000) *Effects of repeat fenbendazole treatment in dairy calves with giardiasis on cyst excretion, clinical signs and production.* Veterinary Parasitology, 89: pp209–218
- O'Handley, R.M., Cockwill, C., McAllister, T.A., Jelinski, M., Morck, D.W. & Olson, M.E. (1999) *Duration of naturally acquired giardiasis and cryptosporidiosis in dairy calves and their association with diarrhoea.* Journal of the American Veterinary Medical Association, 214: p391–396.
- O'Handley, R.M., Olson, M.E., Fraser, D., Adams, P. & Thompson, R.C. (2000). *Prevalence and genotypic characterisation of Giardia in dairy calves from Western Australia and Western Canada.* Veterinary Parasitology, 90: pp193–200.
- Olson, M.E. O'handley, R.M., Ralston, B.J., McAllister, T.A & Thompson, R.C.A. (2004) *Update on Cryptosporidium and Giardia infections in cattle.* TRENDS in parasitology, 20: pp185-191
- Olson, M.E., Ryan. M.O., Ralston, B.J., McAllister, T.A. & Thompson, R.C.A. (2004) *Update on Cryptosporidium and Giardia infections in cattle.* TRENDS in parasitology, 20: pp185-191.
- Olson, M., Goh, J., Phillips, M. & McAllister, T.A. (1999) *Giardia Cyst and Cryptosporidium Oocyst Survival in Water, Soil and Cattle Feces.* Journal of Environmental Quality, 28: pp1991-1996.
- Ons.gov.uk. (2016). *Annual Mid-year Population Estimates- Office for National Statistics*. Available at: http://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/popula

tionestimates/bulletins/annualmidyearpopulationestimates/2015-06-25 [Accessed 9 Jun. 2016].

- Plutzer, J., Ongerth, J & Karanis, P. (2010) *Giardia taxonomy, phylogeny and epidemiology: Facts and open questions* International Journal of Hygiene and Environmental Health, 213: pp321–333
- Palmer, C.S., Traub, R.J., Robertson, I.D., Devlin, G., Rees, R., Thompson, R.C.A (2008) *Determining the zoonotic significance of Giardia and Cryptosporidium in Australian dogs and cats.* Veterinary Parasitology, 154: pp142-147.
- Pollock, K.G.J., Smith, H.V. & Young, D. (2005) *Giardia Surveillance in Scotland, 1988-2003.* European Journal of Clinical Microbiology & Infectious Diseases, 24; pp571-573.
- Porter, J.D. Ragazzoni, H.P., Buchanon, J.D., Waskin, H.A., Juranek, D.D & Parkin, W.E. (1988*) Giardia transmission in a swimming pool*. American Journal of Public Health, 78: 659-662.
- Prystajecky, N., Tsui, C.K.M., Hslao, W.W.L., Uyaguari-Diaz, M.I., Ho, J., Tang, P. & Issac-Renton, J. (2015) *Giardia spp. Are commonly Found in Mixed Assemblages in Surface Water, as Revealed by Molecular and Whole-Genome Characterization*. Applied and Environmental Microbiology, 81: pp4827-4834.
- Ralston, B.J., McCallister, T.A. & Olson, M.E. (2003) *Prevalence and infection pattern of naturally acquired giardiasis and cryptosporidiosis in range beef calves and their dams*. Veterinary Parasitology, 114: pp113–122
- Rimhanen-Finne, R., Hanninen, M., Vuento, R., Laine, J., Jokiranta, T.S., Snellman, M., Pitkanen, T., Miettinen, I. & Kuusi, M. (2010) *Contaminated Water Caused the First Outbreak of Giardiasis in Finland, 2007: A Descriptive Study.* Scandanavian Journal of Infectious Diseases, 42: pp613-619.
- Robertson, L.J. (2009). *Giardia and Cryptosporidium infections in sheep and goats: a review of the potential for transmission to humans via environmental contamination*. Epidemiology and Infection, 137: pp913–921.
- Robertson, L.J., Hanevik, K., Escobedo, A.A., Kristine, Mørch & Langeland, N. (2010) *Giardiasis – why do the symptoms sometimes never stop?* Trends in Parasitiology, 26: pp75-82.

- Robertson, L. Hermansen, L., Gjerde, B.K., Strand, E., Alvsvag, J.O. & Langeland, N. (2006) *Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004.* Applied and Environmental Microbiology, 72: pp2212–2217
- Robertson, L.J. (1996) *Severe Giardiasis and Cryptosporidiosis in Scotland, UK*. Epideniology and Infection, 117: pp551-561.
- Roxstrom-Lindquist, K., Palm, D., Reiner, D., Ringqvist, E. & Svard, S.G. (2006) *Giardia immunity – an update*. TRENDS in Parasitology, 22: pp26-31.
- Ryan, U & Cacciò, S.M (2013) *Zoonotic Potential of Giardia.* International Journal for Parasitology, 43: pp943-956.
- Santin, M., Trout, J.M. & Fayer, R. (2009) *A longitudinal study of Giardia duodenalis genotypes in dairy cows from birth to 2 years of age.* Veterinary Parasitology, 162: pp40-45.
- Savioli, L., Smith, H. & Thompson, A. (2006) *Giardia and Cryptosporidium join the 'Neglected Diseases Initiative'.* TRENDS in Parasitology. 22: pp203-208.
- Schuurman, T., Lankamp, P., van Belkum, A., Kooistra-Smid, M. & van Zwet, A. (2007) *Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of Giardia lamblia in human stool specimens*. Clinical Microbiology and Infection, 13: pp1186-1191.
- Slifko, T.R., Smith, H.V. & Rose, J.B. (2000) *Emerging Parasite Zoonoses Associated with Water and Food.* International Journal for Parasitology, 30; pp1379-1393.
- Smith, A., Reacher, M., Smerdon, W., Adak, G.K., Nichols, G. & Chalmers, R.M. (2006) *Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003.* Epidemiology and Infection, 134: pp1141-1149.
- Smith, H.V., Cacciò, S.M., Cook, N., Nichols, R.A.B. & Tait, A. (2007) *Cryptosporidium and Giardia as foodborne zoonoses*. Veterinary Parasitology, 149: pp29-40.
- Smith, H.V. & Mank, T.G. (2011f) *Diagnosis of Human Giardiasis*. In: Lujam. H.D. & Svärd, S. (Eds) *Giardia: A model organism.* SpringerWeinNewYork. Pp359.

- Smith, H.V., Robertson, L.J., Gilmour, R.A., Morris, G.P., Girdwood, R.W.A. & Smith, P.G. (1993) *The occurrence and viability of Giardia cysts in Scottish raw and final waters.* Water and Environment Journal, 7: pp632-635.
- Solaymani-Mohammadi, S. & Signer, S.M. (2010) *Giardia duodenalis: The double-edged sword of immune responses in giardiasis*. Experimental Parasitology, 126: pp292-297.
- Sprong, H. Cacciò, S.M. & van der Giessen, J.W.B. (2009) *Identification of Zoonotic Genotypes of Giardia duodenalis.* PLoS Neglected Tropical Diseases, 3: pp1-12.
- Stark, D., van Hal, S., Marriott, D., Ellis, J. & Harkness, J. (2007) *Irritable bowel syndrome: A review on the role of intestinal protozoa and the importance of their detection and diagnosis.* International Journal for Parasitology, 37: pp11-20.
- Stark, D., Roberts, T., Ellis, J.T., Marriott, D. & Harkness, J. (2014) *Evaluation of the EasyScreen™ Enteric Parasite Detection Kit of the detection of Blastocystis spp., Cryptosporidium spp., Dientamoeba fragilis, Entamoeba complex, and Giardia intestinalis from clinical stool samples.* Diagnostic Microbiology and Infectious Disease, 78: pp149-152.
- Stiles, C. W. (1902). *The type species of certain genera of parasitic flagellates, particularly Grassi's genera of 1879 and 1881*. Zoologische Anzeiger; 25 pp689.
- Stuart, J. M., H. J. Orr, F. G. Warburton, S. Jeyakanth, C. Pugh, I. Morris, J. Sarangi, & G. Nichols (2003). *Risk factors for sporadic giardiasis: a case-control study in southwestern England*. Emerging Infectious Diseases, 9: pp229–233.
- Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A. & Xiao, L. (2003). *Triosephosphate isomerase gene characterization and potential zoonotic transmission of Giardia duodenalis.* Emerging Infectious Diseases, 9: pp1444–1452.
- Thompson, A.R.C. (2000) *Giardiasis as a re-emerging infectious disease and its zoonotic potential.* International Journal for Parasitology, 30: pp1259-1267
- Thompson, A.R.C., Hopkins, R.M. & Homan, W.L. (2000) *Nomenclature and Genetic Groupings of Giardia Infecting Mammals.* Parasitology Today, 16: pp210-213.

- Thompson, A.R.C., Lymbery, A.J. & Meloni, B.P. (1990) *Genetic variation in Giardia Kunstler, 1882: taxonomic and epidemiological significance.* Protozoological Abstracts, 14: pp1-28.
- Thompson, R.C., Lymbery, A.J., Pearce, D.A., Finn, K.C. Reynoldson, J. A & Meloni, B.P. (1996) *Giardia duodenalis: exposure to metronidazole inhibits competitive interactions between isolates of the parasite in vitro.* Journal of Parasitology, 82: pp679-683.
- Thompson, A.R.C. & Monis, P.T. (2004) *Variation in Giardia: implications for taxonomy and epidemiology*. Advances in Parasitology, 58: pp69–137
- Thompson, A.R.C & Monis, P.T (2011) *Taxonomy of Giardia Species*. In: Lujam. H.D. & Svärd, S. (Eds) *Giardia: A model organism*. SpringerWeinNewYork. Pp8.
- Thompson, A. R.C. & Monis, P.T (2011b) *Taxonomy of Giardia Species*. In: Lujam. H.D. & Svärd, S. (Eds) *Giardia: A model organism.* SpringerWeinNewYork. Pp25.
- Thompson, A.R.C., Morgan, U.M., Mellor, K.J. & Hopkins, R.M. (1999). *Genotyping Giardia and Cryptosporidium.* Today's Life Science, pp1180–1186.
- Thompson, A.R.C (2004) *The zoonotic significance and molecular epidemiology of Giardia and Giardiasis.* Veterinary Parasitology, 126: pp15-35.
- Trout, J.M., Santín, M. & Fayer, R. (2008) *Detection of assemblage A, Giardia duodenalis and Eimeria spp. in alpacas on two Maryland farms*. Veterinary Parasitology, 153: pp203–208.
- Trout, J.M, Santin, M. & Fayer, R. (2007) *Prevalence of Giardia duodenalis genotypes in adult dairy cows.* Veterinary Parasitology, 147: pp205-209.
- Tumova, P., Kulda, J. & Nohýnková, E. (2007) *Cell division of Giardia intestinalis: Assembly and disassembly of the adhesive disc, and the cytokinesis.* Cell Motility and the Cytoskeleton, 64: pp288–298.
- Uehlinger, F.D., Barkema, H.W., Dixon, B.R., Coklin, T. & O'Handley, R.M. (2006) *Giardia duodenalis and Cryptosporidium spp. in a veterinary college bovine teaching herd.* Veterinary Parasitology, 142: pp231–237.
- UK Environment Agency (2010). *Methods for the isolation, identification and enumeration of Cryptosporidium oocysts and Giardia cysts.* The United Kingdom

Environment Agency Blue Book publications. Microbiology of Drinking Water: Part 14.

- Upton, S.J & Zien, C.A. (1997) *Description of a Giardia varani-like Flagellate from a Water Monitor, Varanus salvator, from Malaysia*. Journal of Parasitology, 83: pp971-971.
- Volotao, A.C.C., Souza Júnior, J.C., Grassini, C., Peralta, J.M. & Fernandes, O. (2008) *Genotyping of Giardia duodenalis from Southern Brown Howler Monkeys (Alouatta clamitans) from Brazil.* Veterinary Parasitology, 158: pp133–137.
- Waters, E.K., Hamilton, A.J., Sidhu, H.S., Sidhu, L.A. & Dunbar, M. (2016) *Zoonotic Transmission of Waterborne Disease: A Mathematical Model.* Bulletin of Mathematical Biology, 78: pp169-183.
- Weitzel, T., Dittrich, S., Mohl, E. & Jelinek, A.T. (2006) *Evaluation of seven commercial antigen detection tests for Giardia and Cryptosporidium in stool samples.* Clinical Microbiology and Infection, 12: pp656-659.
- Wells, B., Shaw, H., Innocent, G., Guido,S., Hotchkiss, E., Parigi, M., Opsteegh, M., Green, J., Gillespie, S., Innes, E.A. & Katzer, F. (2015) *Molecular detection of Toxoplasma gondii in water samples from Scotland and a comparison between 529bp real-time PCR and ITS1 nested PCR.* Water Research, 87: pp175-181.
- Wells, B., Thomson, S., Ensor, H., Innes, E.A. & Katzer, F. (2016) *Development of a sensitive method to extract and detect low numbers of Cryptosporidium oocysts from adult cattle faecal samples.* Veterinary Parasitology, 227: pp26-29.
- Wensaas, K., Langeland, N. & Rortveit, G. (2009) *Prevalence of recurring symptoms after infection with Giardia lamblia in a non-endemic area.* Scandinavian Journal of Primary Health care, 27: pp12-17
- Woessner, D.J. & Dawson, S.C (2012) *The Giardia median body proten is a ventral disc protein that is critical for maintaining a domed disc conformation during attachment.* Eukaryotic Cell, 11: pp292-301.
- Wohlsen, T., Bates, J., Gray, B. & Katouli, M (2004) *Evaluation of Five Membrane Filtration Methods for Recovery of Cryptosporidium and Giardia Isolates from Water Samples.* Applied and Environmental Microbiology, 70 (4): pp2318-2322.

- Xiao, L. & Fayer, R. (2008) *Molecular characterisation of species and genotypes of Cryptosporidium and Giardia and assessment of zoonotic transmission*. International Journal of Parasitology, 38: pp1239–1255.
- Ye, J., Xiao, L., Ma, J., Guo, M., Liu, L. & Feng, Y. (2012). *Anthroponotic enteric parasites in monkeys in public park, China.* Emerging Infectious Diseases, 18: pp1640–1643.